

**ANALYSIS OF THE ROLE OF p7 PROTEIN FUNCTION IN HEPATITIS C  
VIRUS LIFE CYCLE**

By © Ali Atoom

A thesis submitted to the School of Graduate studies in partial fulfillment of the  
requirements for the degree of

**Doctor of Philosophy / Immunology and Infectious Diseases / Faculty of Medicine**

Memorial University of Newfoundland

**May 2014**

St. John's      Newfoundland and Labrador

## **ABSTRACT**

The hepatitis C virus genome encodes a 63 amino acid protein, p7. The exact role of p7 is unknown; in this study we observed that mutations in transmembrane domain-1 and -2 (TM1 and TM2), and the cytoplasmic loop of p7 decreased infectious virus production. Analysis of p7 at different stages of virus assembly revealed that p7 functions at a stage prior to generation of infectious particles. Confocal microscopy analyses indicated that p7 did not affect recruitment of core protein to lipid droplets. Additionally, mutation of p7 did not affect formation of core multi-order structures. Finally, mutations at the cytoplasmic loop significantly reduced intracellular E2 glycoprotein levels. Forced evolution analysis of p7 mutations resulted in the occurrence of a N765D mutation that was important for secretion of virus particles into the culture supernatants. The results of this study provide strong evidence that p7 functions to protect HCV glycoproteins from premature degradation and we suggest that p7 supports the virus assembly process.

## ACKNOWLEDGMENTS AND DEDICATION

Firstly, I would like to express my sincere gratitude to my advisor Dr. Rodney Russell for his patience, motivation and continuous support during these past five years. His guidance helped me not only in my research and writing of this thesis, but also he taught me how to be an independent researcher and provided me with much advice during my graduate school career. He helped me academically and emotionally and he gave me the moral support and the freedom I needed to move on. I was lucky to be part of his lab and he was the one who was there for me all the time. His enthusiasm and love for teaching is contagious, I could not have imagined having a better advisor and mentor for my PhD. There are no words to convey how much I am grateful to him.

Besides my advisor, I would like to express my deepest appreciation to my co-advisor Dr. Thomas Michalak and my committee members, Drs. Michael Grant and Joseph Banoub, whose contribution and encouragement helped me to coordinate my project and write this thesis.

Furthermore I would also like to acknowledge with my deepest appreciation for the crucial role of the members in Dr. Russell's lab, Natasha Noel, Nicole Whittle, Kayla Holder, and Heidi Morris. Also, special thanks goes to those who I considered my teammates, Dr. Dan Jones, Nate Taylor, and Hassan Kofahi, who significantly helped me in my PhD with their handy pieces of advice, constructive critique and useful recommendation. I have been touched by the big hearts of Kayla Holder, Chelsea Ash, Megan Conway, and Natasha Noel have I will never forget the respect they showed me. Without them I couldn't have achieved so much alone. I will always remember every motivating chat, and every bit of practical and emotional support by these wonderful people.

I have to appreciate the guidance given by other supervisors in the Immunology and Infectious Diseases Program, including Drs. Vernon Richardson, Sheila Drover, Kensuke Hirasawa, Mani Larijani, Patricia Mulrooney-Cousins and George Carayanniotis, as well as all the technicians, students and postdoctoral fellows in the program, who gave me the permission to use all required equipment and the necessary materials to complete this work. Interaction with them in the program has improved my presentation skills thanks to their comment and advice.

I would like also to acknowledge the advice and guidance of my friends Mussa Suliman, Nader Abosarah, Chris Corkum, and Mohamad Sarhan, who also have been essential parts of my life in Canada. I would not forget to remember my friend Chad who works night shifts in our department. The conversations I have had with him encouraged me to stay working all day in the lab. I would like to thank him for his respect and the time he spent with me, it would have been a lonely lab at night without him.

I recognize that this research would not have been possible without financial support from Memorial University and the Canadian Institutes of Health Research. Their assistance will always be credited and appreciated.

Last but not the least, I would like to thank my hard-working mom, dad, brothers and sisters who sacrificed their lives for me and provided unconditional love and care. I love them so much, and I thank them for their spiritual support during my PhD studies. I would like to thank my wife for her love, kindness and support she has shown during the time she shared with me to finalize this project; she is the one to count on when times are rough, I am very grateful to her for giving birth to my son Ahmad that made the best outcome from these past five years in having a son and a PhD.

Finally I would like to dedicate this work to all of you and to all people in Canada for giving me this opportunity. I hope my education will support me to continue my career in science and benefit our community's health.

## TABLE OF CONTENTS

<b>ABSTRACT .....</b>	<b>i</b>
<b>ACKNOWLEDGMENTS AND DEDICATION .....</b>	<b>ii</b>
<b>LIST OF FIGURES .....</b>	<b>vii</b>
<b>LIST OF TABLES .....</b>	<b>ix</b>
<b>LIST OF ABBREVIATIONS .....</b>	<b>x</b>
<b>CO-AUTHORSHIP STATEMENT .....</b>	<b>1</b>
<b>CHAPTER 1: INTRODUCTION.....</b>	<b>2</b>
1.1 HCV overview .....	2
1.2 HCV discovery and classification.....	3
1.3 HCV genome organization .....	4
1.4 HCV virion structure and physical properties.....	6
1.5 HCV proteins .....	8
1.6 The untranslated regions of HCV .....	13
1.7 HCV life cycle .....	14
1.7.1 HCV entry .....	14
1.7.2 HCV fusion .....	15
1.7.3 HCV genome translation and post-translation modification.....	17
1.7.4 HCV genome replication .....	18
1.7.5 HCV assembly process and release .....	19
1.8 HCV pathogenesis and immune-evading mechanisms .....	25
1.8.1 HCV pathogenesis .....	25
1.8.2 Mechanisms of immune evasion in HCV infection .....	29
1.9 HCV therapy .....	31
1.10 The HCV protein p7.....	35
1.10.1 HCV p7 processing, topology, and localization .....	35
1.10.2 Ion channel function and potential anti-p7 compounds .....	39
1.10.3 NMR analyses of p7.....	45

1.10.4 Functional analyses of p7.....	47
1.10.5 p7 interactions with other viral proteins .....	51
1.11 Project design and hypothesis .....	54
1.12 Research objectives.....	57
<b>CHAPTER 2: MATERIALS AND METHODS .....</b>	<b>58</b>
2.1 Cell culture.....	58
2.2 Cloning and plasmid construction .....	58
2.3 <i>In vitro</i> transcription and RNA transfection .....	59
2.4 Antibodies .....	59
2.5 Indirect IF.....	60
2.6 Virus titration .....	60
2.7 Titration of intracellular infectious virus .....	61
2.8 SDS-PAGE and WB analysis .....	61
2.9 Confocal microscopy .....	61
2.10 Iodixanol density gradient fractionation .....	62
2.11 Bafilomycin A1 and ammonium chloride treatments.....	63
2.12 Forced evolution assay.....	63
2.13 Reverse transcription, PCR amplification and sequencing of rescued mutants.....	64
2.14 Flow cytometric analysis of intracellular core protein.....	64
<b>CHAPTER 3: RESULTS (EFFECT OF p7 ON INFECTIOUS VIRUS PRODUCTION).....</b>	<b>67</b>
3.1 Generation of p7 mutations.....	67
3.2 Effect of p7 mutations within TM1 and the cytoplasmic loop on infectious virus production using single-cycle assay.....	67
3.3 Effect of TM1 and the cytoplasmic loop mutations on infectious virus production using Huh-7.5 cells .....	69
3.4 Effect of TM2 mutations on infectious virus production using Huh-7.5 cells .....	75
<b>CHAPTER 4: RESULTS (ANALYSIS OF THE ROLE OF p7 IN THE HCV LIFE CYCLE) .....</b>	<b>78</b>

4.1 Analysis of intracellular and extracellular species of virus particles.....	78
4.2 Mutations in p7 TM1 and the cytoplasmic loop do not affect core sedimentation profiles .....	79
4.4 p7 TM1 and the cytoplasmic loop do not affect core targeting to LD.....	81
4.5 Mutation of the p7 cytoplasmic loop results in a time-dependent reduction of E2 levels .....	88
<b>CHAPTER 5: RESULTS (FORCED EVOLUTION ANALYSIS OF p7 MUTANTS)</b>	<b>99</b>
5.1 Passaging of p7 mutants led to adaptation and restoration of virus production by acquisition of amino acid changes on different viral proteins .....	99
5.2 The adaptive mutation N765D is important for p7 function.....	100
5.3 Additional analysis of N765D revealed a key role of p7 in efficient viral assembly and provided evidence against involvement in HCV entry processes. ....	108
<b>CHAPTER 6: DISCUSSION .....</b>	<b>113</b>
<b>REFERENCES.....</b>	<b>128</b>

## LIST OF FIGURES

Figure 1.1 HCV genome structure and its translation and processing.....	5
Figure 1.2 HCV virion structure. ....	7
Figure 1.3 HCV life cycle. ....	16
Figure 1.4 Suggested model of HCV assembly and release. ....	21
Figure 1.5 p7 topology and sequence analysis.....	38
Figure 2.1 Illustrative diagram of the different strains of JFH-1 used in this study. ....	65
Figure 3.1 Construction of p7 mutants. ....	70
Figure 3.2 Single cycle virus production assay analysis of p7 mutant viruses. ....	72
Figure 3.3 The p7 cytoplasmic loop and TM1 are important for virus production. ....	74
Figure 3.4 The p7 TM2 is important for virus production.....	77
Figure 4.1 Extracellular particle production and comparison of intracellular vs. extracellular infectious virus. ....	82
Figure 4.2 Iodixanol gradient analysis of p7 mutant viruses using lysis buffer with detergent.....	83
Figure 4.3 Comparison of iodixanol gradient separation of core species, for JFH1 <sub>D</sub> using different conditions. ....	84
Figure 4.4 Iodixanol gradient analyses of p7 mutant viruses after lysis using freeze/thaw method.....	85
Figure 4.5 Analysis of core/LD association.....	87
Figure 4.6 p7 mutants in the background of JFH1 <sub>D</sub> -FLAG showed a similar pattern of virus infectivity as JFH1 <sub>D</sub> virus. ....	89
Figure 4.7 Effects of p7 mutation on FLAG-E2 levels in S29 and Huh-7.5 cells. ....	90



Figure 4.8 Comparison of virus production from JFH1 <sub>D</sub> , JFH1 <sub>S</sub> and JFH1 <sub>A4S</sub> . ....	92
Figure 4.9 Effects of p7 mutations on E2 levels. ....	93
Figure 4.10 Examination of HCV proteins under different lysis conditions. ....	95
Figure 4.11 Time course of E2, core, and GAPDH expression assessed by WB. ....	96
Figure 4.12 Effect of Bafilomycin A1 and NH <sub>4</sub> Cl treatment on E2 levels.....	98
Figure 5.1 A schematic representation of the method used to identify compensatory mutations. ....	101
Figure 5.2 p7 mutant passaging. ....	103
Figure 5.3 Effect of the N765D adaptive mutation on infectious virus production.....	106
Figure 5.4 Effect of the N765D adaptive mutation on infectious virus production for all of the generated p7 mutants. ....	107
Figure 5.5 Illustrative diagram for the constructs selected to analyze N765D adaptive mutation effect on extracellular HCV particles secretions. ....	110
Figure 5.6 Analysis of transfection efficiencies by WB and flow cytometry.....	111
Figure 5.7 N765D increased the production of extracellular HCV particles.....	112
Figure 6.1 Proposed model of p7 functions. ....	123

## LIST OF TABLES

Table 1.1 HCV proteins .....	9
Table 1.2 Examples of known viroporins .....	41
Table 2.1 Primers used to generate p7 mutations .....	66
Table 5.1 Sequence analysis of the rescued p7 mutants .....	104

## LIST OF ABBREVIATIONS

°C	Degrees Celsius
ΔGDD	Negative control with NS5B active site removed
μg	microgram
μL	microliter
μm	micrometer
aa	Amino acids
ADRP	Adipose differentiation-related protein
Apo-A/B	Apolipoprotein-A/B
Bcl-xl	B-cell lymphoma-extra large protein
BSA	Bovine serum albumin
BVDV	Bovine Viral Diarrhea Virus
CD81	Cluster of Differentiation 81
cDNA	Complementary deoxyribonucleic acid
cLD	Cytosolic lipid droplet
CLND1	Claudin-1
CM	Complete medium
CO <sub>2</sub>	Carbon dioxide
cPLA2	Cytosolic phospholipase A2
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CypA or B	Cyclophilin A or B
D1, D2, D3...etc.	Domain1, Domain2, Domain3....etc.
DAAs	Direct-acting antivirals
DAPI	4',6-diamidino-2-phenylindole
DGAT1	Diglyceride acyltransferase 1
dH <sub>2</sub> O	Deionized water
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
E1	Envelope protein 1
E2	Envelope protein 2
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EHM	Extra-hepatic manifestation
eIF3	Eukaryotic translation initiation factor 3
EM	Electron microscopy
EMCV	Encephalomyocarditis virus
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complex required for transport
FBS	Fetal bovine serum
FDA	Food and Drug Administration

G1, G2, G2...etc.	Genotype 1, Genotype 2, Genotype 3.....etc
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBV-B	GB virus B
GFP	Green fluorescent protein
ffu	Focus-forming units
h	Hour/Hours
HA-tag	Human influenza hemagglutinin-tag
HAV	Hepatitis A Virus
HBV	Hepatitis B Virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
HRS	horseradish peroxidase
hSGK1	human serine/threonine kinase-1
HTAs	Host-targeting agents
Huh-7.5	Human hepatoma 7.5
hVAP-A	Human vesicle-associated membrane protein-associated protein A
HVR	Hypervariable region
IF	Immunofluorescence
IFN	Interferon
IRES	Internal ribosome entry site
IRF-3	Interferon regulatory factor-3
ISGs	Interferon stimulated genes
JFH1	Japanese fulminant hepatitis 1
kDa	kilodalton
KIRs	Killer inhibitory receptors
LD	Lipid droplet
LDL	Low density lipoprotein
LVR	Lipoviral particle
MAVS	Mitochondrial antiviral-signaling protein
MHC	Major histocompatibility complex
MICA	MHC Class I Polypeptide-Related Sequence-A
miR-122	MicroRNA-122
mL	Milliliter
mg	Milligram
MTP	Microsomal triglyceride transfer protein
NaCl	Sodium chloride
NF- $\kappa$ B	Nuclear factor- $\kappa$ B
ng	Nanogram
NH <sub>4</sub> Cl	Ammonium chloride
NK cells	Natural Killer cells
NMR	Nuclear magnetic resonance
NS	Non-structural protein

NTR	Nontranslated region
OCLN	Occludin
ORF	Open reading frame
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononucleated cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death-1
PEG-IFN	Pegylated interferon
PenStrep	Penicillin Streptomycin
PKR	Protein kinase R
PRK	Protein kinase C-related kinase
PRR	Pattern recognition receptors
RdRp	RNA-dependent, RNA-polymerase
RIG-I	Retinoic acid-inducible gene 1
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcription-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SR-B1	Scavenger receptor B type-1
SVR	Sustained virological response
TAE	Tris base, acetic acid and EDTA buffer
TGF- $\beta$	Transforming growth factor- $\beta$
TLR-3	Toll-like receptor-3
TM	Transmembrane
TMD	Transmembrane domain
TNF $\alpha$	Tumor necrosis factor $\alpha$
TRIF	Toll/interleukin-1 receptor -domain-containing adapter-inducing- interferon- $\beta$
UTR	Untranslated region
UV	Ultraviolet
VLDL	Very low density lipoproteins
VLP	Viral lipoparticles
WB	Western blot
WHO	World Health Organization

## CO-AUTHORSHIP STATEMENT

All research described in this thesis was performed in the laboratory of Dr. Rod Russell. Chapter 1 contains a general introduction regarding HCV, followed by a more focused review of the literature available on HCV p7. This latter portion of this Introduction is now in press at *Virology* and I am listed as first author since I wrote 100% of this review, with editorial contributions from my lab colleague, Nathan Taylor, and my supervisor.

Results described in Chapters 3 and 4 have been published in *Virus Research* with me as first author as I performed 90% of the experiments. A colleague in the lab, Dr. Daniel Jones, helped with the core sedimentation assay development and contributed to the writing and revision of the manuscript. All experiments were designed by me and my supervisor.

The results described in Chapter 5 have been submitted for publication at *Virology Journal*. I performed 80% of the experiments described, participated in the design of the study and co-drafted the manuscript. Nathan Taylor participated in the design of primers and cloning of p7 mutants and co-drafted the manuscript. Hassan Kofahi performed the Flow Cytometry analysis of intracellular core staining.

Over the course of my PhD program I also contributed to two other publications from our team, including a second authorship on a *Journal of Virology* paper from Dr. Jones, and a 4<sup>th</sup> authorship on a paper to be submitted to *PLoS Genetics*, by Heidi Morris.

## **CHAPTER 1: INTRODUCTION**

### **1.1 HCV overview**

Hepatitis C virus (HCV) is the major cause of several severe liver diseases including chronic hepatitis, liver fibrosis, cirrhosis, and hepatocellular carcinoma; often resulting from complications of persistent HCV infection. These disorders can lead to liver transplantation to treat liver failure in infected individuals [1]. The estimated rate of HCV infection around the world is 2.6-3.1% representing 122-185 million symptomatically infected individuals [2]. Unfortunately, there is no prophylactic vaccine and the current combinations of pegylated interferon- $\alpha$  (Peg-IFN $\alpha$ ) and ribavirin are expensive and associated with severe side effects [3,4]. This combination has been reported to be only successful for approximately 50% of individuals infected with HCV genotype-1 (G1). Treatment success is defined as the achievement of undetectable HCV RNA by currently available clinical laboratory assays for at least 6 months after treatment and termed a sustained virological response (SVR). Interestingly, 80% of patients infected with G2 or G3 and 60% of patients infected with G4 achieve such a clinically defined SVR [4,5]. In 2011, two direct-acting antiviral compounds (DAAs) targeted to the virally encoded protease (NS3-4A) became available as part of a new triple therapy approach in combination with Peg-IFN $\alpha$  and ribavirin. This breakthrough led to improvement of the SVR rate from approximately 50% to 70% for G1-infected patients [6,7]. However, this new strategy was limited to certain genotypes (G1 and G2) and associated with viral resistance mutations, and increased drug toxicity and side effects [8]. Therefore, efforts

are ongoing to develop improved treatment options. A better understanding of basic HCV virology will doubtlessly impact the identification of novel therapeutic targets.

## **1.2 HCV discovery and classification**

Early in the 1970s, an unknown etiological agent that was neither hepatitis A virus (HAV) nor hepatitis B virus (HBV) (non-A non-B) was considered to be the major cause of parental acquired and post-transfusion hepatitis [9]. In 1989, the Chiron group succeeded in partial-genome cloning and development of an antibody detection system for diagnosis of infection with this unknown virus, which was then called HCV [10,11]. Consequently, the full length HCV genome was characterized and found to be a positive-sense single-stranded RNA of approximately 9.6 kb in length [12-14]. The genome organization, and HCV viral precursor proteins, as well as the morphological features of HCV particles taken from human serum and liver tissue were shown to be congruent with that of flaviviruses and pestiviruses in the *Flaviviridae* family [15-18]. However, because of the low sequence homology of HCV with that of flaviviruses and pestiviruses, HCV was classified in a distinct genus termed hepacivirus [19]. Subsequently, the genus included the enigmatic GB-virus B and the newly-identified non-primate, rodent and bat hepatitis viruses (NPHV, RHV and BHV) [20-22].

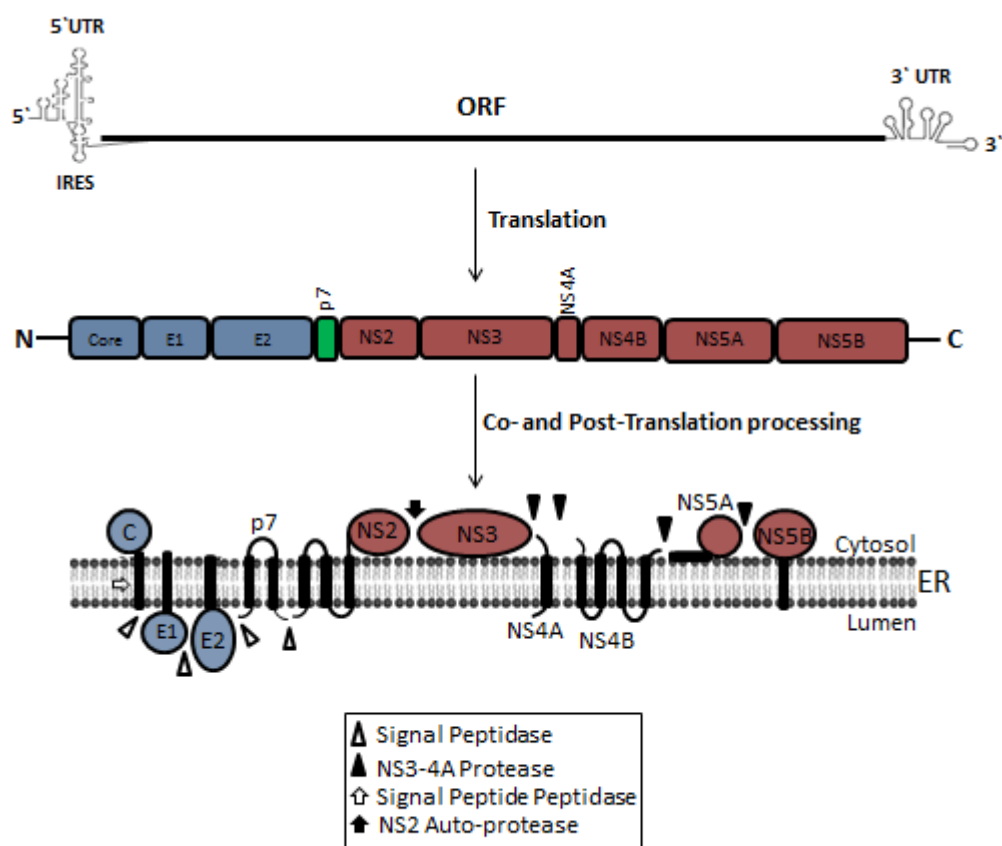
There are at least 7 genotypes of HCV (G1-G7) that differ by approximately 30% in their nucleotide sequence [23]. These genotypes are further classified into subtypes (a, b, c, d, *etc*). The variations are attributed to the high mutation rate of HCVs error-prone RNA polymerase and diverge in worldwide distribution, transmission, and disease progression [24]. As another repercussion of the error-prone polymerase, HCV circulates



as a diverse population of closely related variants in infected individuals, referred to as a quasispecies [25].

### **1.3 HCV genome organization**

Since the elucidation of the full length HCV genome, it became understood that the positive-sense RNA genome encodes a long polyprotein precursor encoded by an open reading frame (ORF) of approximately 3010 amino acids (aa) with minor genotypic variation (3008-3037 aa) [26]. Processing of the polypeptide chain occurs in a co- and post-translational manner, with cleavages catalyzed by cellular and viral proteases to generate the mature forms of each individual protein (Figure 1.1) [27-29]. The 1<sup>st</sup> third of the genome encodes the viral proteins core, and envelope glycoproteins 1 and 2 (E1 and E2), which are the structural components of the virion [26]. Downstream of these proteins is the viral protein, p7, which is dispensable for RNA replication, but its role in the virion assembly process is becoming of further interest to the research community [30,31] and the subject of this thesis. The remaining two thirds of the genome encode the non-structural proteins (NS) in the order: NS2, NS3, NS4A, NS4B, NS5A and NS5B. The non-structural proteins act to catalyze polypeptide cleavages, form replication complexes and perform crucial roles in the assembly process [26,32]. The HCV ORF is flanked by 5' and 3'-untranslated regions (5'-UTR and 3'-UTR). The 5'-UTR (~341 nucleotides) is highly conserved due to its possession of an internal ribosomal entry site (IRES) [33] that mediates genome translation [34]. The 3'-UTR (~139 nucleotides) most likely functions during initiation of genome replication [13,35].



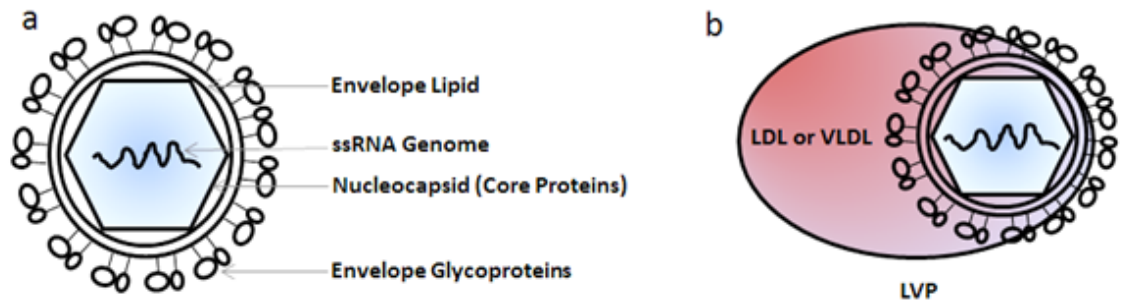
**Figure 1.1 HCV genome structure and its translation and processing.** The HCV genome (top panel) contains an approximately 9.6 kb open reading frame (ORF) flanked by 5'- and 3'-untranslated regions (UTRs). Translation is initiated at the IRES sequence within the 5'- TR to generate a 3000 amino acids polypeptide chain comprised of unprocessed structural proteins core and envelope glycoproteins 1 and 2 (blue boxes; middle panel), as well as the non-structural proteins 2-5B (red boxes). The viral protein p7 is neither a structural nor non-structural protein (green box). The polypeptide undergoes co- and post-translational processing in the endoplasmic reticulum (ER) to generate the mature forms of the viral proteins (enzymatic digestion sites are indicated by symbols (bottom panel) as defined in the insert).

#### **1.4 HCV virion structure and physical properties**

Based on the better characterized virion structural arrangement of the related flaviviruses, it has been suggested that HCV particles are enveloped with a host-derived lipid membrane bi-layer, and display surface viral glycoproteins E1 and E2 in complex (Figure 1.2a). Beneath the envelope resides the viral nucleocapsid that consists of an oligomerized version of core and encases a single copy of the viral RNA genome [36]. Electron microscopic visualization of HCV particles derived from patient serum and liver tissue showed a spherical shape particle of 50 to 70 nm in diameter [15-17].

The nucleocapsid is 50 nm in diameter and icosahedral in structure [16]. The buoyant density of HCV particles derived from patient serum is low and heterogeneous (1.04-1.13 g/ml), which is unusual for enveloped RNA viruses [37]. Serum-derived HCV particles were immunoprecipitated by using antibodies to apolipoprotein-E (Apo-E) and apolipoprotein-B (Apo-B), interacting with Apo-A1, ApoB-48, Apo-B100, and Apo-E, suggesting association of circulating HCV particles with serum low density lipoproteins (LDL) and very low density lipoprotein (VLDL) [38-40]. These features along with immunoglobulin bound circulating particles might explain such unusual heterogeneous density distribution (see section 1.7.5).

Conversely, cell culture-derived (HCVcc) particle densities range between 1.0 and 1.18 g/ml and interact with Apo-E, but Apo-B interaction was varied [41-45]. The lipid profile of HCVcc particles revealed a similar lipid and cholesterol composition to serum-derived particles. Despite this similarity, the apolipoprotein class in cell culture could not be consistently defined [42]. The discrepancies between serum and HCVcc-



**Figure 1.2 HCV virion structure.** (a) Model of hepatitis C virus particle. (b) Model for the lipoviral particle (LVP) in which the virion is associated with LDL or VLDL.

derived particles can be attributed to a defect in the Huh7 cell lines ability to secrete Apo-B containing VLDL particles [46].

The association of HCV particles with serum lipoproteins suggests that HCV particle synthesis relies on cellular lipoprotein formation and HCV virions form a hybrid lipoviral particle (LVP) (Figure 1.2b). This hybrid formation might protect circulating particles from antibody neutralization or lysis in an extra-hepatic environment, as well as facilitate virus entry into hepatocytes [47]. However, the exact mechanism of association is still unclear. Further details on the correlation between HCV formation and cellular lipid synthesis are discussed subsequently in this thesis.

### **1.5 HCV proteins**

Core protein is the building block of the viral nucleocapsid and can be divided into two domains: D1 and D2. D1, formed by the N-terminal two-thirds of core, is highly hydrophobic and contains multiple positively charged amino acids suggested to be important for viral RNA binding and homo-oligomerization of core [48,49]. D2 forms the C-terminal third of core protein and is important for association with lipid droplets (LD); the primary site of HCV nucleocapsid assembly. This association is required for virus production in cell culture [48,50]. Expression of core protein alone in transgenic mice leads to steatosis and development of hepatocellular carcinoma (HCC) [51,52]. It has been suggested that core protein upregulates fatty acid biosynthesis in Huh7 cells and interacts with Apo-AII. This research leads to the intriguing indication that core plays a major role in pathogenesis and development of liver steatosis and HCC via deposition of triglycerides in the liver [50] (HCV proteins with the known and putative up to date functions are listed in Table 1.1).

**Table 1.1 HCV proteins**

<b>Viral protein</b>	<b>Approximate nt/aa length</b>	<b>Molecular mass (kDa)</b>	<b>Function</b>
Core	573/191 (mature form is 173-179 aa)	21	RNA binding, nucleocapsid formation, upregulation of lipid metabolism
E1	576/192	33	Envelope formation, mediation of viral entry
E2	1089/363	70	Envelope formation, mediation of viral entry and antibody escape
p7	189/63	7	Ion channel, virus assembly
NS2	651/217	21	Virus assembly
NS3	1893/631	69	NS viral protein processing, inhibition of innate immunity and helicase activity
NS4A	162/54	6	NS3 cofactor
NS4B	783/261	27	Membranous web formation
NS5A	1341/447	56-58	Genome replication and assembly
NS5B	1773/591	68	Viral polymerase

Viral proteins listed with their approximate nucleotide (nt) and amino acid (aa) length shown in the 1<sup>st</sup> and 2<sup>nd</sup> columns, respectively. The molecular mass in kilo-Dalton (kDa) as observed in SDS-PAGE analysis is listed in the 3<sup>rd</sup> column, and up to date known and putative function are listed in the last column.

Envelope glycoproteins E1 and E2 are structural components of the HCV particles that form non-covalent complexes and are required for virus entry via receptor binding. E1 and E2 contain N-terminal ectodomains and C-terminal hydrophobic membrane anchors [53,54]. A critical post-translation N-linked glycosylation and disulfide bond formation is required for heterodimer complex formation and host receptor binding [53,54].

E2 binds to the host tetraspanin receptor protein CD81 and scavenger receptor-B1 (SR-B1) found on the surface of hepatocytes and facilitates the entry process [55,56]. Furthermore, E2 was shown to possess hypervariable regions termed HVR-1 and HVR2 that continuously change in patients with HCV infection. The HVRs are significant in viral escape from neutralizing antibodies [57].

p7 is an integral membrane protein that has been shown to form ion channel activity and is suggested to be important for virus assembly (reviewed in [58]). p7 is the major protein of interest in this work, and as such will be described in details following the general introduction (section 1.10).

NS2 is a transmembrane autoprotease that is composed of two domains: an N-terminal membrane anchor and a C-terminal cysteine protease, which cleaves itself from the adjacent NS3 protein after translation [28,59]. NS2 is required for HCV infectivity *in vivo* and in HCV cell culture system (HCVcc), but is dispensable for viral RNA replication [60,61]. It has been shown that NS2 homodimerizes and can interact with other viral proteins. These interactions have led to the suggestion that NS2 has a major role in the HCV assembly process [62-66].

NS3 is composed of an N-terminal serine protease and a C-terminal RNA helicase [67,68]. NS4A protein functions as a cofactor to optimize NS3 protease activity and ER targeting. NS4A contributes to proper folding of NS3 and stabilizes the protease to prevent its degradation [69,70]. NS3 with its cofactor, NS3-4A, is responsible for polyprotein processing of the non-structural proteins either independently, or autocatalytically in tandem with NS2 [71]. Additionally, NS3-4A induces proteolysis of Mitochondrial antiviral-signaling protein (MAVS) and Toll/interleukin-1 receptor-domain-containing adapter-inducing-interferon- $\beta$  (TRIF) adaptor proteins that leads to inhibition of the retinoic acid inducible gene-1 (RIG-I) pathway and Toll-like receptor-3 signaling, respectively, thereby inhibiting an innate immune response by blocking IFN production [72-75]. The C-terminal helicase of NS3 binds RNA with a high affinity and unwinds RNA helices in an ATP-dependent manner [76,77]. The role of RNA unwinding in the life cycle has yet to be fully elucidated. Multiple theories have been developed: (i) it may be important for RNA replication through unwinding of secondary structures or stable stem-loops at the termini of the viral RNA genome [78]; (ii) it could be important for HCV assembly and RNA genome transfer and packaging into the newly-formed nucleocapsid [32,79].

NS4B is an ER-associated protein composed of two amphipathic helices at the N-terminal domain, 4 transmembrane (TM) segments at the central domain, and a palmitoylated C-terminal domain containing two  $\alpha$ -helices [80]. NS4B induces ER membrane alteration forming a ‘membranous web’ during infection. This membrane manipulation suggests that NS4B is crucial for the establishment of replication complexes [81]. Intriguingly, NS4B has RNA binding capability and ATPase/GTPase activity, but



the requirement for such activities within the HCV life cycle remains controversial [82-84].

NS5A is a multifunctional protein involved in HCV replication and assembly. NS5A is a zinc-binding metalloproteinase protein and has no TM domains. The protein can be divided into 3 domains. The N-terminal domain (D1) of NS5A has a conserved RNA binding groove and is important for membrane attachment and LD targeting [85-87]. The second domain (D2) is less conserved and important for viral genome replication [87]. D2 of NS5A has been shown to inhibit interferon-induced double stranded RNA activated protein kinase R (PKR), and inhibit IFN-gamma production [88,89]. In other studies, the D2 domain of NS5A was shown to interact with the host protein cyclophilin A to support viral genome replication [90-92]. Cyclosporin inhibits HCV replication by blocking the interaction between NS5A and cyclophilin A [90]. The C-terminal domain (D3) is also less conserved and required for virus production, but dispensable for RNA replication [93,94]. NS5A has been detected in basal and hyper-phosphorylated forms. It has been suggested that NS5A is required for delivery of newly synthesized RNA genomes to the site of nucleocapsid formation at the surface of LDs, and that phosphorylation status maintains the balance between RNA replication and assembly [94-96]. This notion was supported by the finding that mutations reducing the levels of hyper-phosphorylated form led to a significant enhancement in RNA replication [97,98]. In transgenic mice, NS5A was shown to inhibit innate and adaptive immune responses [99]. Altogether, these studies highlight the importance of this protein at multiple stages of viral morphogenesis and justify NS5A as a current target for development of antiviral therapy. The drug daclatavir is one typical example of NS5A inhibitors that showed rapid decline of HCV

RNA in both cell culture and serum of infected patients, thereby providing a promising future treatment for HCV infection [100].

NS5B is the well-characterized viral RNA-dependent RNA-polymerase that mediates RNA genome amplification. NS5B is anchored to the ER membrane via its first 21 C-terminal amino acids [101]. The crystal structure of the protein revealed typical finger, palm and thumb subdomains. The active site possesses the GDD amino acids motif where the finger and thumb subdomains guide the RNA to the active site through tunnel formation [102,103]. NS5B generates a high error-rate during replication due to its lack of a proofreading mechanism (reviewed in [104]).

### **1.6 The untranslated regions of HCV**

The ORF of HCV is flanked by 5'- and 3'-UTRs that contain essential elements for HCV translation and replication. The 5'-UTR possesses the IRES sequence that initiates cap-independent translation of the viral genome via binding to host cell ribosomes. The IRES contains 4 RNA stem-loops termed I to IV of which domain IIIId constitutes the binding site for the 40S subunit of host ribosomes [34]. The host noncoding RNA element termed microRNA-122 (miR-122) was shown to interact with the 5'-UTR and modulate HCV translation [105]. The abundance of miR-122 was directly proportional to HCV translation efficiency, highlighting the importance of miR-122 as a host factor target for HCV therapy [106].

The 3'-UTR of HCV initiates positive-strand synthesis and stimulates IRES-dependent translation of the viral genome [107,108]. It forms three different domains including a short variable region, a poly U/UC domain, and a highly conserved 3'-X tail [109]. The

poly U/UC was found to be part of the HCV-associated pathogen associated molecular pattern (PAMP) recognized by the innate immunity receptor RIG-I [110].

## **1.7 HCV life cycle**

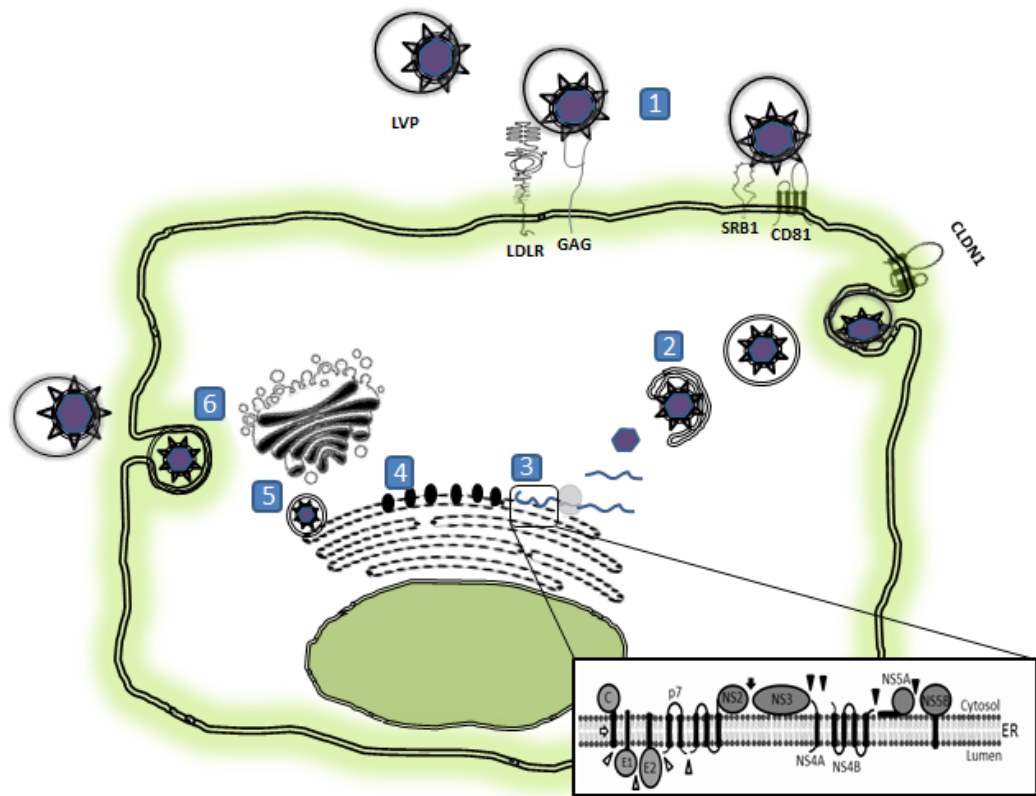
### **1.7.1 HCV entry**

Multiple receptors have been reported to be involved in HCV entry into hepatocyte-derived cell lines such as Huh-7.5 (Figure 1.3). Initial low-affinity attachment is mediated by LDL and glycosaminoglycan (GAG) receptors found on the surface of host liver cells [111,112]. Because HCV circulates in the blood as LVPs, both receptors are thought to interact with the virion-associated ApoE. This speculation has been supported by the finding that antibody blocking and LDL receptor knockdown reduced HCVcc infection [113]. Subsequently, CD81 and SRB1 receptors mediate tight binding of HCV to infected cells [114,115]. CD81 directly binds to the HCV E2 protein and primes HCV E2 for low pH-dependent fusion [116]. Antibodies against CD81 and SRB1 were shown to inhibit HCV infection after attachment, substantiating the involvement of CD81 and SRB1 after initial binding of HCV to host cells [117-119]. The tight junction proteins OCLN and CLDN1 are also important for HCV entry at a post-binding step [120,121]. OLCN and CLDN1 do not bind directly to E2, but CLDN1 was shown to be important for virus entry through its interaction with CD81 [122]. Engineered mice expressing human OCLN and CD81 were found to be permissive to HCV infection, but the exact contribution of OCLN in virus entry is still under investigation [123,124]. Auxiliary receptor including epidermal growth factor receptor (EGFR) and ephrin receptor type A2 have been implicated in the stabilization of the CD81-CLDN1 interaction [125].

The internalization of HCV occurs through clathrin-mediated endocytosis; a process leading to association of the virion with endosomal vesicles inside the host cell [126]. In summary, HCV entry seems to be a sequential process mediated by stepwise receptor engagement. This cascade of receptor interactions and conformational changes leads to virion internalization. Considering this, HCV liver tropism is likely determined by the collective association of these receptors on the hepatocyte (reviewed in [32,127]).

### **1.7.2 HCV fusion**

Fusion is considered one of the least understood steps in the life cycle of HCV. The fusion process in related viruses, such as tick borne encephalitis virus, is well known and the fusion peptide within the E protein has been identified [128]. In pestiviruses, the fusion peptide resides in the E1 protein [129,130]. Therefore, some have proposed that HCV may possess an analogous mechanism of fusion. The viral envelope fusion with the endosomal membrane requires low pH-endosomes to induce viral genome release into the cytoplasm [131,132]. Multiple reports have suggested that a class-II fusion protein resides within the HCV E1 protein [133,134]. Others have argued that the fusion activity resides in E2 protein [135]. Therefore, the mechanisms of fusion and uncoating of internalized HCV particles remain unsolved and need to be fully characterized.



**Figure 1.3 HCV life cycle.** HCV entry is mediated by a sequential binding to multiple receptors that leads to internalization of the virus by clathrin-mediated endocytosis (1). Then, pH dependent fusion and release of the HCV genome take place after dissociation of the HCV-associated clathrin coated vesicles (2). Subsequently, the IRES mediates ORF translation and formation of a polyprotein precursor that is then processed to generate individual mature forms of the viral proteins (3). Viral RNA replication occurs within a modified ER membrane termed the membranous web (4). Then, viral assembly takes place at ER-associated LDs (5) and mature virus is released from the infected cell through a cellular secretory pathway (6).

### 1.7.3 HCV genome translation and post-translation modification

Once the HCV genome is freed from the capsid within the cytoplasm of infected cells, viral translation initiation takes place at the ER surface by formation of the translation complex in a multistep process including: (i) formation of a binary complex between the HCV IRES and the 40S ribosomal subunit; (ii) assembly of a 48S complex at the AUG initiation codon with the eukaryotic translation initiation factor 3 (eIF3), GTP and Met-tRNA<sup>met</sup>, and (iii) hydrolysis of the GTP and joining of the 60S subunit forming the IRES-80S complex [109].

Translation of the HCV ORF yields a polyprotein precursor that is co- and post-translationally processed to liberate the mature form of each individual viral protein (Figure 1.1 and Figure 1.3). The mature structural proteins are further processed by host-encoded signal protease cleavage [27,136,137]. Additional processing at the C-terminal end of core protein is needed to generate the mature form of this protein and is mediated by host signal peptide peptidase cleavage [138]. The cleavage efficiency at the E2-p7 and p7-NS2 junctions is poor, suggesting the existence of functional precursor sequences [137,139].

The NS2 protein autocatalytically cleaves itself at the junction between NS2 and NS3 by the activity of its cysteine protease domain [140]. The second virally-encoded protease NS3, along with its co-factor NS4A, cleaves the remaining non-structural proteins [141,142]. The NS3-4A serine protease cleaves at the NS3-NS4A site in a *cis*-acting manner, whereas the cleavages at the downstream sites are carried out in a *trans*-acting manner [143-145]. More importantly, the cysteine residues at the cleavage sites are conserved in all trans-cleavage sites, as mutations therein impaired cleavage and

processing of NS proteins [146]. In summation, HCV precursor protein processing by host and viral encoded proteases yields 10 different HCV proteins in the following order NH<sub>2</sub>-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH.

#### **1.7.4 HCV genome replication**

The aim of viral genome replication is to amplify genetic material to be packaged in the newly formed virions during the morphogenesis cycle. All positive-strand RNA viruses perform RNA replication in a distinctive altered membrane structure that can be derived from ER, Golgi, mitochondria or lysosome (reviewed in [147]). The altered membrane structure formed in HCV-infected cells was found in the ER and was named the membranous web, which is most likely induced by the viral protein NS4B [81,148]. The membranous web forms distinct factories composed of viral proteins, altered cellular proteins and replicating RNA that altogether form the replication complex [148].

The RNA-dependent RNA polymerase enzyme (NS5B) uses the positive strand as a template to synthesize a negative strand intermediate. Subsequently, the enzyme uses the negative strand as a template to synthesize positive strands [102]. NS3 possesses a helicase domain that could be important for unwinding secondary RNA structure or displacement of RNA-binding proteins in support of genome replication [149]. Additionally, NS5A D1 and D2 were shown to be essential for RNA replication [87,150]. However, the exact contributions of NS3 and NS5A to RNA replication still need to be conclusively identified. It remains unclear how NS5B activity is regulated during RNA replication, but multiple hypotheses have been proposed including: (i) NS5A binds NS5B to modulate its activity [102]; (ii) the flexible beta-hairpin loop at the thumb domain was shown to regulate the RNA binding and initiation of RNA synthesis [151], and (iii) the

regulation of NS5B activity can be mediated by multiple host intracellular factors, such as sphingomyelin, cyclophilin A and B (CyPA and CyPB), and the NS5A binding partner, protein kinase C-related kinase 2 (PRK2), and human vesicle-associated membrane protein-associated protein A (hVAP-A) [152-156]. However, the exact mechanism of regulation and the contributions of the above-mentioned host factors in HCV infection are still not clear.

### **1.7.5 HCV assembly process and release**

After viral protein maturation and RNA replication, an organized assembly process brings together nascent virion components. The following section describes a putative stepwise process of virus assembly that is also shown in Figure 1.4.

#### **1.7.5.1 Trafficking of core to LD**

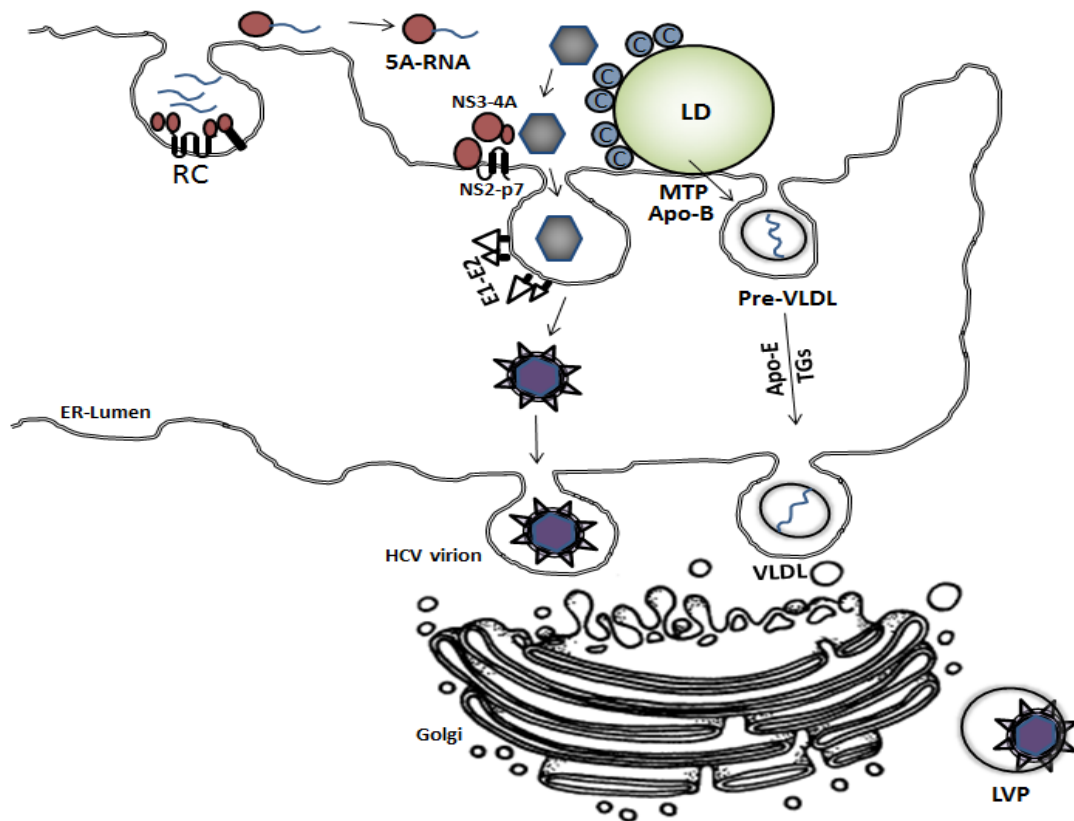
The viral assembly process is assumed to begin with accumulation of core protein at the surface of cellular organelles termed lipid droplets (LD) (Figure 1.4) [157]. Cholesterol esters and neutral lipids are stored in the cytosolic LD (cLD) that are surrounded by a phospholipid monolayer derived from the outer leaflet of the ER [158]. The aim of core trafficking to cLD is not clear but it has been hypothesized that this process sequesters the core protein until it's needed for virus assembly and that the surface of cLD can subsequently serve as a platform for nucleocapsid assembly [47]. The D2 domain of the core protein has implicated importance in accumulation of core around LD because mutations in D2 inhibit this process and significantly abrogate virus production [48]. Furthermore, core protein dislocates a surface LD resident protein called adipose differentiation-related protein (ADRP) and leads to LD redistribution toward the nucleus in a microtubule- and dynein-dependent manner.



This redistribution was suggested to enhance encounters between the core and the replication complex during assembly [159]. Trafficking of core to LD also requires a mitogen-activated protein kinase called cytosolic phospholipase A2 enzyme (cPLA2) and diglyceride acyltransferase 1(DGAT1). Inhibition of the activity of cPLA2 or DGAT1 inhibits virus production [160,161].

#### **1.7.5.2 Nucleocapsid formation and genome encapsidation**

After accumulation of core around LD, core protein oligomerizes and binds viral RNA through its basically-charged domain D1 to form the nucleocapsid structure [49]. It is still unclear how the newly formed nucleocapsid packages the RNA genome. One possibility is that the delivery of viral RNA to the site of nucleocapsid formation and packaging is occurring via NS5A [47]. Consistent with this, NS5A was found to accumulate around LD through its N-terminal amphipathic helix and bind RNA through its zinc-binding domain [85,162]. Another potential explanation is that NS5A is acting only to deliver the viral RNA genome to the site of assembly and that the NS3-4A helicase activity binds RNA and serves to package it during nucleocapsid formation [32]. This notion was supported by the findings that the NS2 or NS2-p7 complex binds NS3 and this interaction mediates colocalization of these proteins to a site in close proximity to cLD [65,66]. Adding weight to this statement, it was found that mutations in the NS3 helicase domain enhancing RNA replication cause defects in virus assembly, highlighting a role of NS3 in the assembly process as a possible mediator of RNA packaging [32,163].



**Figure 1.4 Suggested model of HCV assembly and release.** HCV is thought to initiate assembly in close association with LD. NS5A acts to deliver HCV RNA genomes to the site of assembly. Nucleocapsid RNA packaging is suggested to be accomplished by the viral protein NS3-4A complex. Consequently, the assembled capsid then buds into the lumen of the ER where glycoproteins E1/E2 reside. The orchestrating viral protein NS2 serves to bring non-structural and structural proteins together to the site of assembly in collaboration with p7. The newly formed virion is secreted through the secretory pathway passing the Golgi network and the formed virion associated with VLDL particles is secreted out of the cell as viral lipoparticle (VLP).

### 1.7.5.3 Nucleocapsid envelopment

A late step in the assembly process is the membrane envelopment of the newly-formed nucleocapsid. Multiple studies have indicated that this step is tightly linked to the VLDL pathway [37,44,164-166]. VLDL formation starts with ApoB-100 synthesis through insertion of phospholipids and triglycerides from the cLD to the luminal side of ER membrane through the activity of the microsomal triglyceride transfer protein (MTP). This will lead to formation of a spherical like structure, termed the luminal LD (LLD), which then incorporates more triglyceride and Apo-E molecules to form mature VLDL. The mature VLDL's then move to a distal compartment in the secretory pathway and are released from the cell (reviewed in [167]). HCV formation is highly linked to this pathway due to the following observations: (i) fractionation of HCVcc-infected cells showed that HCV particles were found in detergent-resistant lipid fractions and were rich in similar VLDL lipid compositions [164,165]; (ii) HCV particles in infected patients were found to circulate as LVPs that are rich in triglycerides, and immuno-capture studies suggest that they contain Apo-B, Apo-E, and Apo-C along with the viral proteins core, E1 and E2 [37,44,45,166,168]; (iii) chemical inhibition of VLDL pathway components, such as MTP, cPLA2 and DGAT1, as well as genetic silencing of Apo-E and Apo-B, significantly inhibits virus production [44,160,161,169], and (iv) most significantly, HCV particles in live cell imaging were found to colocalize with Apo-B in close proximity to LD and traffic with Apo-E to the cell periphery [170]. Taken in whole, the preceding evidence demonstrates the close association of HCV morphogenesis with lipid metabolism and suggests the assembly process likely hijacks the VLDL synthesis pathway.

The question remains: how are the envelope glycoproteins targeted to the assembly site and incorporated into the newly formed virion? E1 and E2 proteins form non-covalent heterodimers that are retained in the ER lumen [171]. It was shown that NS2 alone or the p7-NS2 complex modulates colocalization of the E1-E2 complex and NS3-4A to LD by direct or indirect interactions [65,172,173]. Interestingly, a study investigating core protein trafficking in live virus-producing cells found that during the peak of virus assembly, core formed LD-independent puncta adjacent to the putative sites of assembly that traffic along microtubules [174]. Importantly, core was recruited from LDs into these puncta, and interaction between the viral NS2 and NS3-4A proteins was essential for this recruitment process [174]. Therefore, NS2-p7 might be orchestrating this step by interacting with NS3-4A in one manner to bring newly formed capsids to the site of assembly and with the E1-E2 complex in another way to facilitate the envelopment step (this model was suggested in [32]).

#### **1.7.5.4 Virus budding and release**

The virion forms by budding from the ER and egresses from the cell through the secretory pathway and is presumably released in a VLDL-dependent manner [175]. E1 and E2 contain high mannose and complex N-linked glycans; these types of modifications are indicative of passage through the Golgi [176]. Supporting this idea, envelope proteins rearrange the disulphide bonds necessary to prime HCV particles for low pH-mediated fusion, similar to pestivirus egress through the Golgi [131,176,177]. The trafficking of HCV particles pre-envelope maturation is thought to be within intracellular vesicles. At this stage, the viral protein p7 ion channel activity was shown to protect nascent virions from low pH-mediated inactivation [178]. Contributing to this idea, a RNAi study

implicated SAR1A (a GTPase protein involved in membrane trafficking and found in COPII vesicles) in the production of extracellular HCV, suggesting HCV particles transport from the ER to the Golgi with COPII secretory vesicles [170]. Subsequently, virions are passaged from Golgi to the plasma membrane also through the secretory pathway as inhibition of the PI4KB (resident Golgi lipid kinase that is important in trafficking from the Golgi to the plasma membrane) significantly reduced the average velocity of motile labelled core puncta using live cell imaging [170]. Moreover, movements of the cytoplasmic labelled core puncta were dependent on NS2 function [170]. This evidence suggests a model of HCV trafficking that is highly dependent on the cellular secretory pathway and leads to acquisition of additional pH-resistance modifications in the viral envelope. Characterization of the envelopment process remains elusive, but the roles of p7 and NS2 have become increasingly conspicuous.

Finally mature virions exit infected cells through the secretory pathway. The endosomal sorting complex required for transport (ESCRT) pathway is involved in membranous compartment fission that curves away from the intracellular cytosol [179]. This pathway is utilized by multiple enveloped viruses [179-182]. However, other studies showed that HCV particles are transported to the cell membrane through recycling endosomes that fuse with the plasma membrane to release virions into the extracellular milieu [170,183]. This could indicate that the ESCRT pathway is required for virus particle budding from the ER or virus trafficking into an intermediate secretory compartment. Endosome contributions may facilitate HCV particle movement to a cell junction protein domain and facilitate cell-to-cell spread [32,170,183]. Notably, the tight junction protein claudin 1 has been reported to be required for HCV cell-to-cell

transmission [184]. More analysis is needed to identify HCV release pathways and the possibility that HCV might spread through both extra-cellular release and cell-cell spread must be considered.

## **1.8 HCV pathogenesis and immune-evading mechanisms**

### **1.8.1 HCV pathogenesis**

HCV-infected patients mainly acquire HCV infection by blood contact and, rarely, through sexual transmission. Thus, the main high risk groups are intravenous drug users, health care workers, and blood transfusion recipients before 1992 [185]. Within approximately 21 days after infection HCV RNA becomes detectable in serum [186,187]. Anti-HCV antibody detection normally occurs 32-46 days after viremia [188]. A small minority of patients spontaneously clear the virus to an undetectable level by the currently available clinical assays, while the majority (54-86%) develop chronic hepatitis [189]. Jaundice is the specific clinical sign of acute infection, but some patients develop nonspecific signs, which can include fatigue, nausea, mild fever, abdominal pain and loss of appetite [190]. The majority of patients remain asymptomatic during the acute phase of infection and, unfortunately, they do not become aware of the disease until much later in life [190].

It is still unclear why some patients eradicate the infection during the acute phase of the disease, but several factors have been postulated which include: (i) gender and age, as it was shown that women resolve acute hepatitis C two times more often than men and acquiring infection at a younger age might facilitate viral clearance [190,191]. However, the underlying mechanisms are not clear and controversial findings have been published [192,193]; (ii) a strong HCV-specific CD8<sup>+</sup> T cell response is correlated with the control

of viremia during the acute phase of infection [194]; (iii) also, natural killer (NK) cells from HCV-infected patients, but not from healthy controls, overexpress inhibitory receptors and produce cytokines that attenuate the adaptive immune response, such as transforming growth factor- $\beta$  (TGF- $\beta$ ) and interleukin-10 (IL-10) [195]. Genetic factors appear to contribute to NK cell activity. Allelic variation impacts expression of killer inhibitory receptors (KIRs) on NK cells and HLA expression on target cells, both of which play key roles in NK cell activation [196]. Furthermore, a high rate of self-limiting disease is associated with the presence of clinical symptoms during the acute phase of the disease and suggested that a stronger immune response arises in symptomatic patients [197], and (iv) certain single nucleotide polymorphisms (SNPs) in the *IL28B* gene that encodes interferon-lambda-3 were found to greatly contribute to both spontaneous clearance of HCV infection and response to interferon-based therapy [198,199]. However, the mechanism of IL28B SNPs in viral clearance and treatment response is still not clear.

The chronic phase of the disease can be defined as a persistent infection in which HCV RNA remains detectable for longer than six months after transmission [200]. Chronic hepatitis C is accompanied by liver damage and may result in liver fibrosis, cirrhosis, and HCC [201]. It was assumed that liver injury during the chronic stage is immune-mediated due to the fact that T cell infiltration in the liver temporally correlates with the onset of liver injury [201]. However, rapid disease progression in immunodeficient patients or with therapeutic immunosuppression infers that additional factors are involved in liver injury [202]. In the HCVcc system, it was found that HCV replication induced cell death-related genes and caused apoptosis [203]. This notion was

supported by a study performed in chimeric mice with humanized liver and lacking an adaptive immune system (SCID/Alb-uPA), which also found that apoptosis was induced by HCV infection [204]. The observed apoptosis in SCID/Alb-uPA HCV-infected mice was mediated by a combination of induction of ER and oxidative stress and downregulation of the anti-apoptotic proteins nuclear factor- $\kappa$ B (NF- $\kappa$ B) and B-cell lymphoma-extra large (Bcl-xl) [204]. Analysis of liver tissue from patients with chronic viral hepatitis demonstrated upregulation of human serine/threonine kinase-1 (hSGK1) and signaling mediators of transforming growth factor- $\beta$  (Smad), both of which are apoptosis markers and associated with TGF- $\beta$  signaling [205,206]. Taken together, these findings suggested that HCV infection not only induces liver cell apoptosis, but also can induce fibrosis by activating TGF- $\beta$  signaling, which lead to collagen deposition [207].

As disease progresses, fibrous bridges form between adjacent portal areas, which can lead to cirrhosis in around 15-56% of the patients with chronic HCV infection [208,209]. Activated liver stellate cells produce excessive amounts of extracellular matrix including collagen and are the main contributors in the development of cirrhosis [210]. HCC develops at a higher rate in cirrhotic HCV-infected individuals, but still can be detected in non-cirrhotic patients with advanced fibrosis [211]. The mechanism of HCC is poorly understood, but dysregulation of cell proliferation by HCV replication is probably essential for the immortalization of infected hepatocytes. It also was suggested that hepatocyte regeneration in an oncogenic environment containing mutagens, such as oxidants, which are present as a consequence of cell injury, fibrosis and immunological attack, can result in the emergence of HCC [212]. The HCV core protein was shown to transform cells into a malignant phenotype through cooperation with transforming protein



p21 (H-ras) [213]. It has also been reported that other HCV proteins, such as NS3, NS4B and NS5A, have oncogenic potential, but the mechanism of involvement in cellular transformation and *in vivo* relevance remains to be defined [214-216]. Risk factors can also increase the incidence of HCC development, including co-infection with hepatitis B virus (HBV) or Epstein-bar virus (EBV) [217-219]. Genetic background is also another risk factor as a SNP in MHC class I polypeptide-related sequence-A (MICA) was strongly associated with the onset of HCC in HCV-infected patients [220]. All of the above, as well as other risk factors have been proposed, but the evidence is less clear and there is a lack of representative data.

Chronic HCV infection may also cause extra-hepatic manifestations (EHM) with 40-74% of infected individuals shown to develop at least one EHM [221,222]. Mixed cryoglobulinemia (MC) is the most commonly associated disorder with HCV infection at a rate of 19-55% [223]. Other EHMs were also reported, including lymphoma, glomerulonephritis, vasculitis, insulin resistance and type 2 diabetes, and autoimmune disorders, such as thyroiditis, depression, anemia and impaired renal function [223-225]. One interesting observation is that HCV can also infect cells of the immune system [226-228]. HCV RNA positive and negative strands, as well as viral proteins have been visualized in isolated peripheral blood mononuclear cells (PBMC) from chronically infected patients [226,228]. Multiple reports have described reactivation of HCV infection after SVR in immunological disorders such as HIV or hypogammaglobulinaemia [229,230]. In addition, immunosuppressive drug receipt has also been shown to associate with recurrent HCV infection despite an apparent SVR [231,232]. This idea is also supported by the above-mentioned association of HCV with

MC and non-Hodgkins lymphoma. HCV variants isolated from immune cells were different from those in serum or liver in infected individuals and from the JFH-1 strain obtained from HCVcc [233-236]. These differences were mapped to the 5'-UTR and promote HCV replication in immune cells but not in liver cells [237]. As a whole, these observations suggest that HCV infection of immune cells could be responsible for reactivation of HCV after SVR and HCV may undergo a latency-like stage during the course of infection or treatment period in what's called occult HCV infection (OCI) [238]. However, the exact mechanism and role of OCI in disease progression and reactivation is still not clear and requires more investigation. Such findings are most important to understand mechanisms of HCV reactivation after apparently successful treatment.

### **1.8.2 Mechanisms of immune evasion in HCV infection**

HCV has evolved several mechanisms to establish a persistent infection. The first line of defense that HCV evades is the innate immune response (reviewed in [239]). The viral protein NS3-4A was shown to cleave mitochondrial antiviral signaling protein (MAVS) both in cell culture systems and in infected patient hepatocytes [240]. MAVS is a part of a pattern recognition receptor (PRR) pathway that is important for sensing viral RNA and mounting an immune response [241]. RIG-I senses the pathogen associated molecular pattern (PAMP) in the HCV genome and activates a signaling pathway through MAVS leading to production of downstream effector molecules, such as interferon regulatory factor 3 (IRF-3), NF- $\kappa$ B and a variety of pro-inflammatory cytokines required for innate antiviral immunity and IFN production [241]. NS3-4A was also shown to degrade TRIF and inhibit RNA sensing through the RNA sensing PRR termed toll like receptor 3 (TLR-

3) [75]. TLR-3 and its signaling through the adaptor protein TRIF activates IRF-3 and NF- $\kappa$ B for the production of IFN and inflammatory cytokines needed for proper immune system function, such as upregulation of the cytotoxic T cell response [242]. The viral proteins NS5A and E2 were shown to inactivate protein kinase R (PKR), an example of a non-traditional PRR that senses viral dsRNA [243-245]. NS5A and E2 can inhibit the kinase-dependent activity of PKR and lead to phosphorylation of eukaryotic initiation factor 2 alpha to suppress host mRNA translation, but not HCV translation [243-245]. NS5A and E2 suppress PKR kinase-independent activity as well, acting to inhibit induction of specific IFN-stimulated genes (ISGs) and IFN- $\beta$  by signaling through MAVS before RIG-I activation [88,243-245]. However, HCV inhibition of PKR kinase-dependent activity could limit host factors important for HCV replication. Therefore, it was suggested that NS5A and E2 PKR kinase-dependent inactivation functions at a specific time during infection to support availability of required host factors for HCV replication after NS3-4A-mediated inhibition of MAVS [239]. The core protein of HCV also contributes to the inhibition of innate immune responses by antagonizing IFN signaling through blockage of the Jak-STAT signaling pathway [239,246,247].

HCV can also evade components of the adaptive immune system directly. The high rate of HCV replication combined with the polymerase lack of proofreading helps the virus to escape the antibody response [248,249]. HCV escape mutations also inhibit CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses by affecting epitope processing, MHC binding, and recognition by both CD8<sup>+</sup> and CD4<sup>+</sup> T cells [248,250-252]. Fascinatingly, HCV-specific T cells isolated from blood and liver biopsies of infected patients express the inhibitory receptor programmed cell death-1 (PD-1), which inhibits effector functions and

accelerates apoptosis upon engagement with its ligand expressed on infected hepatocytes [253,254]. The effector function of HCV-specific T cells isolated from infected patients' blood can be rescued by *in vitro* exposure to PD-1 blocking antibody and after blocking of cytotoxic T-lymphocyte antigen 4 (CTLA-4) and PD-1 for T cells isolated from liver biopsies [254,255]. Studies on HCV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells found them to be impaired in their effector functions, such as cytotoxicity and production of effector mediators IL-2, TNF- $\alpha$  and IFN- $\gamma$  [256-259]. The mutation rates and amino acid changes in HCV was found to be affected by immune responses, with the highest level of selective pressure peaking during the acute phase of the disease and decreasing gradually as the infection continues [248,251,260]. Therefore, T cell accumulation with a sequential loss of function could be a result of altered peptide ligands that downregulate T cell responses and fail to effectively prime new T cells during the course of infection leading to persistence of the pathogen [252,261].

## **1.9 HCV therapy**

Until recently, the standard treatment for HCV infection consisted of Peg-IFN- $\alpha$  and ribavirin [262]. Peg-IFN- $\alpha$  is a general antiviral agent supporting the immunological response, whereas ribavirin activity is not well defined [263]. Multiple mechanisms have been reported for the activity of ribavirin that include: induction of IFN-stimulated genes (ISGs); enhancement of the Th1 immune response; inhibition of HCV NS5B polymerase activity; and GTP depletion by inhibiting inosine monophosphate dehydrogenase [263]. This treatment combination led to a SVR of about 50% for patients infected with G1 and G4, and about 80% for G2- and G3-infected patients [5,264]. Sadly, this treatment combination is associated with severe side effects, including fatigue, flu-like symptoms,

mild anxiety, skin rash, nausea, diarrhea, autoimmune diseases, hemolytic anemia, depression, and other neuropsychiatric side effects [265,266]. True selection of viral resistance to Peg-IFN- $\alpha$  and ribavirin treatment has not been observed.

Since 2011, standard HCV therapy against infection with G1 has been updated to include Peg-IFN- $\alpha$ , ribavirin and one of two DAA compounds targeting the NS3-4A protease in a triple therapy approach [6,7]. The 1<sup>st</sup> generation NS3-4A protease inhibitor telaprevir and boceprevir increase the SVR rate from 50% to 70% but are unfortunately associated with viral resistance and severe side effects [6,7]. A number of other 1<sup>st</sup> generation protease inhibitors, such as simeprevir, vaniprevir, asunaprevir and faldaprevir are in clinical development and showed more antiviral activity with milder side effects [8,267]. These 1<sup>st</sup> generation protease inhibitors, dependent on class, show overlapping resistance profiles and a limited genotype coverage [8]. The 2<sup>nd</sup> generation protease inhibitors MK-5172 and neceprevir showed higher antiviral activity and a broader genotype coverage with favorable resistance profiles. Both are currently under development [268].

Other DAA compounds targeting viral proteins other than NS3-4A were also developed. The nucleoside inhibitor sofosbuvir targets the NS5B polymerase active site and showed 90% SVR as determined by clinical HCV RNA detection assays and satisfactory safety profiles [269,270]. The nucleoside inhibitors have pangenotypic coverage and a high barrier to resistance due to their mechanism of action [267]. NS5A also emerged as a DAA target with the antiviral compound daclatasvir showing the most promise [271]. Daclatasvir showed broad genotypic activity, reduced HCV infectivity with sub-micromolar concentrations in cell-based assays, and rapidly reduced circulating

HCV in clinical trials [271-273]. This compound has a low barrier to resistance [272]. The idea of using daclatasvir in combination with other DAA classes such as sofosbuvir has been tested with promising results and is likely going to be used in combination approaches with other DAAs in the hope that an IFN-free all-oral combination may eventually become the standard of care [274].

One of the strategies used to develop HCV treatment is through targeting cellular proteins involved in HCV propagation inside an infected cell; such compounds are termed host-targeting agents (HTAs) [275]. For example, the small molecule ITX 5061, which inhibits HDL uptake by the liver through the SR-B1 receptor, was shown to inhibit HCV entry and is currently in phase 2 clinical trials [276]. Mutations in E2 HVR-1 can confer resistance to ITX 5061 [277]. Cyclophilin A (CypA) is an intracellular factor found to bind NS5A and important for HCV replication [153]. Alisporivir is a CypA inhibitor that demonstrates potent antiviral activity and enhances IFN-based treatment, but is associated with acute pancreatitis among trial participants and is now on clinical hold [278-280]. Another promising host factor target required for HCV replication is the liver-specific miR-122 [281]. MicroRNAs are small RNA sequences required to reduce the level of host translated proteins through interaction with messenger RNA (mRNA), thus regulating host gene expression [282]. In the case of HCV infection, miR-122 interacts with the HCV genome at the 5'-end and enhances HCV replication [281]. This interaction was also suggested to protect uncapped HCV RNA from degradation by cellular exonucleases and shield the 5'-end of the genome from recognition by PRRs [283,284]. A small complementary sequence antagonizing miR-122 (antagomir or miravirsen)

significantly inhibits HCV viral load after subcutaneous injection of miravirsen in the chimpanzee model and infected humans, and is now in clinical development [285,286].

In addition to DAAs and HTAs, HCV vaccine development is also under way, where such achievement will be of outstanding value towards HCV eradication. HCV neutralization antibodies against E2 protein derived from hyper-immunized rabbits were shown to prevent HCV infection of chimpanzees [287]. However, antibody-based vaccines fail due to HCV mutations and cell-to-cell transmission where virions are hidden from neutralizing antibody contact [184,287]. It remains possible that a cloned pool of antibodies can be used as post-exposure prophylaxis treatment [288]. DNA vectors encoding E1, E2, core, NS3 or NS3-NS5B were also investigated in infected chimpanzees as a strategy to elicit strong T cell responses and develop a protective HCV vaccine [289-292]. However, the observed protection in these vaccination strategies was short in duration and could not prevent HCV infection after rechallenge.

Treatment of HCV improved after the approval of the first two DAAs. A collaborative effort of basic understanding of the HCV life cycle, industrial drug development and clinical investigations provide hope for identifying novel therapeutic agents that can lead to HCV eradication.

## **1.10 The HCV protein p7**

The review of the HCV p7 protein below has been published in *Virology* as an Invited Review.

### **1.10.1 HCV p7 processing, topology, and localization**

The p7 protein was identified through expression of a series of C-terminally truncated HCV polyproteins fused to a human c-myc epitope tag. This analysis located p7 between the envelope glycoprotein E2 and the NS2 protein (Figure 1.1) [137]. Homologous proteins have been identified in the *Pestivirus* genus, e.g., bovine viral diarrhea virus (BVDV), classical swine fever virus, and border disease virus, all containing such a characteristic protein between E2 and NS2 [293]. However, Flaviviruses such as yellow fever virus, dengue virus, and West Nile virus do not encode a protein homologous to p7 [294].

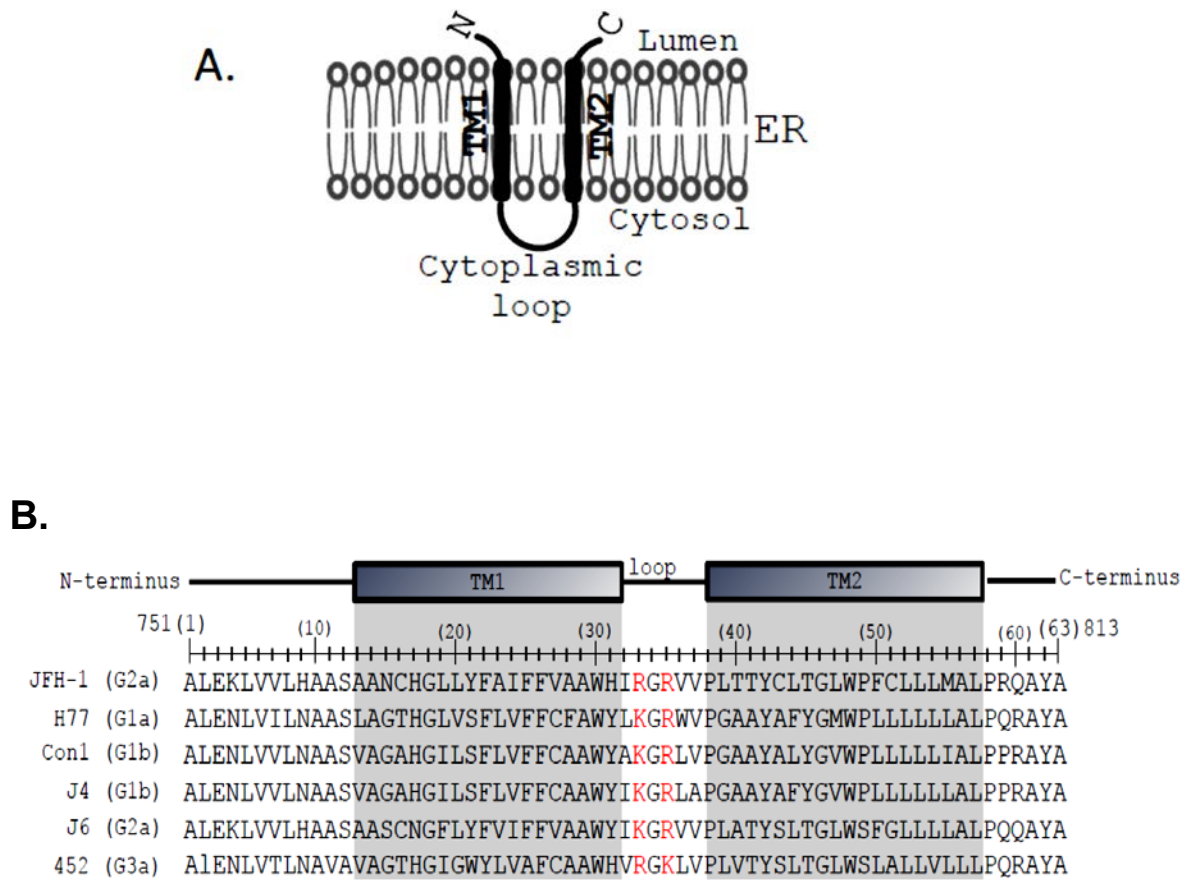
Upon HCV genome translation, p7 processing is mediated by host-encoded proteases (Figure 1.1) [19,295], but scission at the E2-p7 and p7-NS2 junctions is delayed, resulting in the presence of precursor E2-p7-NS2 polyproteins. The cleavage at the E2-p7 junction is incomplete and performed by a unique mammalian signal peptidase leading to the presence of E2 and p7 mature forms, as well as some E2-p7 species [137,139,171]. Partial cleavage was attributed to a structural determinant located N-terminal to the signal peptides. This is supported by the finding that fusion of reporter proteins N-terminal to the signal peptides improves cleavage efficiency [296]. The function of E2-p7 or p7-NS2 precursors is yet unidentified, but hypotheses have been developed: (i) the precursor itself plays a role in the HCV life cycle; (ii) the precursor could be important for regulating the kinetics and/or levels of final product expression to prevent premature virus assembly



[296,297] (such a case was illustrated in the coordinated cleavage of C-preM in the Murray Valley encephalitis virus [298]); (iii) the E2-p7 precursor might be important for pulling p7 into the physical composition of the virion by retaining a pool in the ER as the E2-p7 form [299]. However, it's likely that cleavage of E2-p7 precursors play a regulatory role because complete separation of E2 and p7 by insertion of an encephalomyocarditis virus (EMCV) IRES between these two proteins moderately reduced the level of virus production in the HCVcc system. This suggests the E2-p7 precursor serves no functional purpose on its own, but that a temporal dependence on E2-p7 processing is critical for virus production [297,300]. In trans-complementation studies, it was found that whether p7 alone or p7 in the context of cleavage intermediates were sufficient for restoration of virus production depended on the extent of the original p7 deletion and the degree of indirect effects such deletions had on polyprotein processing. [301]. In summary, it is clear that the p7-including processing intermediates in some way impact virus production, but whether these intermediates have specific functions within the viral life cycle requires further investigation.

The p7 protein is a polytopic membrane protein that crosses the ER membrane twice, forming trans-membrane domain 1 (TM1) and trans-membrane domain 2 (TM2) connected by a short segment (Figure 1.5), with its N- and C-termini oriented toward the cytosol [137]. The subcellular localization of p7 was identified by fusion of p7 to CD4 or Myc. These fusions in HepG2 cells identified a large fraction of p7 in an early compartment of the secretory pathway suggesting the presence of a retention signal maintaining localization of p7 in the ER [302]. Conversely, intracellular staining of green fluorescent protein (GFP) or Flag-tagged p7 in 293T cells showed that p7 partially co-

localized with mitochondria and adjacent membrane structures [303]. Interestingly, staining of native and tagged p7 demonstrated that untagged p7 was exclusively detected on the ER, while N-terminally tagged p7 was detected on ER or mitochondrial adjacent membranes. This work is indicative of complex trafficking of p7 that could be regulated by the cleavage from its upstream signal peptide and targeting signals present within the protein sequence. Transmission of calcium ion fluxes from ER cisternae to mitochondria has been shown to be a pivotal process in the regulation of apoptotic signaling. Therefore, The authors suggested that p7 association with ER and mitochondrial membranes may interfere with such signals, rendering the cell insensitive to proapoptotic stimuli from immune cells or the effects of other viral gene products [299,304]. Immunofluorescence (IF) and electron microscopy (EM) in the context of a full-length, RNA replication-competent HCV genome derived from the JFH-1 sequence, showed that GFP-p7 or p7 fused to human influenza hemagglutinin-tag (HA-tag) 4 amino acids downstream of the potential E2-p7 cleavage site were localized only at the ER in Huh7 cells. However, it is important to note that these tags likely disrupted virus production [305]. Consequently, recent studies have double-HA-tagged p7 at the N-terminus in the chimeric virus Jc1, creating a viable virus. This replicating virus showed significant colocalization of p7 with ER markers and a lesser extent of association with mitochondrial or LD markers. p7 showed a reticular staining pattern that mainly co-localized with HCV E2 and partially with NS2, NS3, and NS5A [306]. Hypothetically, p7 localization with multiple organelles may be indicative of this protein's dual role in HCV assembly and trafficking of nascent virions through multiple cellular pathways.



**Figure 1.5 p7 topology and sequence analysis.** (A) Representative diagram of p7 topology within the ER showing the TM1 and TM2 connected by a cytoplasmic loop. Both N- and C-termini are oriented toward the ER lumen. (B) Examples of commonly used isolates with genotype/subtype sequence variability and domain locations shown. TM1 (aa13-32) and TM2 (aa38-57) are indicated by dark gray boxes. The two conserved basic residues in the cytoplasmic loop are shown in red.

The relatively small size of p7 and its membrane integration topology have made analysis of localization and function laborious and sometimes inconclusive. It is possible that the accessibility of antibodies or the duration of infection chosen in the above-mentioned literature inaccurately identified localization of p7 in multiple cellular compartments. Therefore, more sensitive proteomic analysis for p7 tracking and localization on multiple separated cellular organelles at various times during HCV infection in cell culture is needed in order to gain more reliable insights into the role(s) of p7 in the HCV life cycle.

#### **1.10.2 Ion channel function and potential anti-p7 compounds**

The p7 proteins of BVDV and HCV were originally proposed to oligomerize and form ion channels by Harada *et al.* [307] and Carrere-Kremer *et al.* [302], respectively. Oligomerization was observed when it was shown that p7 formed a hexamer in artificial membranes and functioned as a calcium ion channel in so called black lipid membranes. The ion channel activity of p7 in this assay could be abrogated by the drug amantadine [308]. The ion channel activity of a cross-linked p7 led to inclusion of this protein in the viroporin family, which consists of small hydrophobic proteins with the ability to permeabilize membranes for ion and small molecule movement (examples are listed in Table 1.2 and reviewed in [309-313]). An important example is the viral protein M2 of influenza virus, which forms proton ion channels and is activated in acidic environments. M2 triggers viral uncoating during entry within acidic endosomes and protects the HA glycoprotein from premature maturation in the trans-golgi network during egress, indicating that a multitude of functions can be performed by virally encoded ion channels. The channel activity of M2 is blocked by amantadine and rimantadine, suggesting the two

could be used to treat influenza infection (reviewed in [314,315]). p7 conductance across artificial lipid membranes was confirmed when pentamer formation was shown to be necessary for accommodation of ion transport. This study also reported a concentration-dependent inhibition of p7 channel activity after treatment with long-alkyl-chain iminosugar derivatives, such as *N*-nonyl deoxygalactonojirimycin (NN-DGJ), *N*-nonyl deoxynojirimycin (NN-DNJ) and *N*-7-oxanonyl-6-deoxy-DGJ [316]. These compounds were tested because of their potent anti-viral activity against BVDV [317].

In cell-based assays, Griffin *et al.* demonstrated that expression of p7 in mammalian cells can substitute for influenza virus M2 channel activity to maintain infectivity of influenza, and that this activity also could be blocked by the antiviral drug amantadine. In addition, it also was shown that mutation of the two basic residues within the p7 cytoplasmic loop abrogated ion channel activity, highlighting the importance of this loop for p7 function [303]. A subsequent study confirmed the ion channel function of p7 in planar lipid bilayer membranes and showed that p7 channels were permeable to potassium and sodium ions, with limited permeability to calcium ions [318]. Later, fluorescence-based liposome assays were developed to more conveniently assay for p7 channel activity and therapeutic targets within p7. Employment of such assays using p7 from genotype 1b showed a dose-dependent release of fluorescent indicator when mixed with liposomes. The release activity was blocked by the drugs amantadine, rimantadine and several related compounds [319]. Taken together, these similarities with M2 protein function and inhibition provide strong evidence that p7 is also a viroporin.

**Table 1.2 Examples of known viroporins**

<b>Viroporin name</b>	<b>Virus family</b>	<b>Main function</b>	<b>Oligomerized state/Class</b>	<b>Inhibitors</b>
Influenza A M2	<i>Orthomyxoviridae</i>	1. Viral genome uncoating. 2. Assembly of functional HA conformation.	Tetramer/Class IA	Amantadine and Rimantadine
HIV-1 Vpu	<i>Retroviridae</i>	1. Facilitate budding of newly formed virion. 2. Enhance degradation of CD4.	Pentamer/Class IA	Hexamethylene amiloride, BIT225
Picornavirus P2B	<i>Picornaviridae</i>	Modulate virus release and host cell apoptosis (suggested)	Dimer or tetramer/Class IIB	DIDS (classic anion exchanger inhibitor) tested only in Enterovirus
CoV-E	<i>Coronaviridae</i>	1. Induce assembly by enhancing membrane scission. 2. Induce virion release.	Dimer, tetramer, or pentamer /Class1A	Hexamethylene amiloride

Ion channel names and their respective virus and family names are shown (1st and 2nd columns). The known or expected functions are listed (3rd column). The number of monomers needed to form the channel upon oligomerization and the classification (class) are shown (4th column). Potential inhibitors (5th column). Abbreviations: HA: hemagglutinin, HIV-1: Human immunodeficiency virus type 1, Vpu: viral protein U, CoV: coronavirus. (Table information is gathered from references [309-313]).

Investigation of sequence determinants in p7 critical for ion channel activity or drug sensitivity led to a number of insightful outcomes concerning the role of the putative channels. One report showed that the leucine-rich motif at the C-terminal end of TM2 partially contributed to drug sensitivity. Mutation of the two basic residues within the cytoplasmic loop (K33/R35 in G1b) was found to affect channel activity and caused disruption of the ion channel insertion into membranes. The histidine at residue 17 in TM1 also showed significant effects on ion channel function [320]. H17 is part of a HXXX(Y/W)-like motif that is also found in influenza M2 and is integral for ion channel opening [321]. H17 was shown to be in a critical position in the ion pore, demonstrating its key role in ion channel conductance [322]. Additionally, the L20F mutation and F25A polymorphism in HCV p7 were found to confer adamantane and iminosugar resistance, respectively [323]. These findings highlight the importance of mutational analyses of the p7 sequence and the associated ion channel activity, with major implications on drug binding and potential identification of novel inhibitors.

p7 channel activity had never been tested in a cell culture-based assay recapitulating the entire HCV life cycle because such a system (HCVcc) was not established until 2005 [324-326]. In contrast with earlier studies, discrepancy was observed when the HCVcc system was employed to characterize the potential antiviral effects of amantadine and iminosugar derivatives (NN-DGJ, NN-DNJ and NB-DNJ). It was found that amantadine did not affect virus release, infectivity or ion channel activity of JFH-1 (G2a) or chimeric HCV genomes (H77 (G1a), Con1 (G1b), and J6 (G2a)). In contrast, the iminosugar derivatives reduced virus titers in a dose-dependent manner in the context of multiple genotypes, primarily at virus assembly or release steps [327]. The iminosugar derivatives

NN-DGJ, NN-DNJ and NB-DNJ have been shown also to target cellular  $\alpha$ -glucosidase I and II, and their inhibition leads to misfolding of various host [328] and viral glycoproteins, such as BVDV E2 [329] and gp120 of HIV [330]. With this in mind, the above-mentioned effect of iminosugar derivatives on virus production could be attributed to alteration in HCV envelope proteins, as shown previously [327], or to a manipulation of a host cellular glycoprotein crucial for virus propagation in cell culture. Similar discrepancy was also observed in a related protein called p13-c of GBV-B, whereby amantadine inhibited the function of p13-c ion channel activity but failed to inhibit replication and secretion of GBV-B from virally-infected marmoset hepatocytes [331].

The development of the HCVcc system introduced greater opportunities for p7 characterization. Griffin *et al.* extensively differentiated the genotype- and subtype-dependent sensitivity of p7 to multiple inhibitors using both HCVcc and artificial membrane assays. It was found that p7 from the JFH-1 isolate was not blocked by amantadine in HCVcc or liposome-based assays, whereas amantadine successfully inhibited G3a in both systems. Amantadine inhibits J4 (G1b), while rimantadine was able to inhibit both JFH-1 and J4 channel activity and virus production. In addition, the iminosugar derivatives NN-DGJ and NN-DNJ reduced infectious virus production for genotype 2a and 1b in a dose-dependent manner, with no observed effect on viral protein synthesis, processing, HCV RNA replication or cellular cytotoxicity [332]. Until now, differing results have been obtained with various inhibitors in the context of different genotypes. These contrasting results suggest that p7 inhibitors may need to be developed in a genotype-specific manner.



Interestingly, p7 ion channel activity was identified in native cell membrane vesicles and infected Huh-7.5 cells by measuring  $H^+$  proton conductance and fluorescent detection of lysosomal pH indicators. Similarly, expression of p7 in HEK-293FT cells equilibrated  $H^+$  conductance. HCV infection increased lysosomal pH in infected cells, but defective ion channel mutants yielded no such increase. Furthermore, amantadine, rimantadine and hexamethylene amiloride block  $H^+$  conductance through native vesicular membranes [178]. In a very recent article, Atkins *et al.* provided new evidence suggesting that p7 directly influences the stability of secreted, acid-labile HCV particles via an as yet undefined mechanism [333]. This strongly advocates the importance of ion channel activity during virus propagation.

A small compound termed BIT225 was shown to inhibit HIV production at a late stage in the viral life cycle by blocking Vpu ion channel activity and impeding virus release from monocyte-derived macrophages [334]. BIT225 was also shown to bind p7 using a computational model of a p7 monomer, along with amantadine, rimantadine and NN-DNJ [335]. BIT225 was shown to inhibit p7 ion channel activity in lipid membranes and demonstrated antiviral activity in a BVDV infection assay [336].

A small number of clinical trials that included potential p7 inhibitors have been performed to date. Combination therapy of amantadine and IFN- $\alpha$  exhibited an insignificant correlation between amino acid variation within p7 and response to treatment in G1a/b-infected individuals. However, a L20F mutation was observed more often in non-responder patients infected with G1b receiving this combination therapy, which gives weight to the theory that amantadine targets p7 [337]. Subsequently, an increase in early anti-virological response was observed in G1a/b patients treated with

IFN- $\alpha$ , ribavirin and amantadine, yet sustained virological response rates remained the same as in the arm receiving standard of care [338]. Such early inhibition was observed previously when a combination of amantadine, ribavirin and IFN- $\alpha$  was shown to reduce plasma viral RNA levels in patients in a phase 1 trial [339]. These data suggest that amantadine could be useful in the case of new infections or to promote viral decay before the establishment of chronic infection.

It is still premature to include an ion channel inhibitor with IFN- $\alpha$  and ribavirin. The discrepancies observed among the inhibition studies performed to date could be attributed to system inconsistencies, *e.g.*, artificial membrane, liposome assay or HCVcc, and/or use of dissimilar p7 genotypes studied in native form versus tagged versions of the protein. Clinical trial data is still confined to response rates and lack of some aspects of drug delivery analysis is plausibly accountable for the minimal therapeutic outcomes. Completion of extensive mutational analyses on p7 could further our structural and functional understanding of the ion channel and identify specific regions within p7 that could represent novel therapeutic targets. Subsequent or concomitant analyses of patient genomes from clinical trials could provide penetrative information concerning resistance and susceptibility in the p7 region.

### **1.10.3 NMR analyses of p7**

Nuclear magnetic resonance (NMR) structural analysis performed on p7 from G1b showed that the N-terminal portion formed an  $\alpha$ -helix (aa 1-14) and TM1 (aa 15-18) and TM2 (aa 19-32) formed  $\alpha$ -helices connected by a short loop (aa 33-39) [340]. However, the structural features and the helix lengths observed through NMR conflicted with the molecular model of p7 [302,320,341,342]. The monomeric NMR structure of p7 unveiled

a dynamic nature, and the structure identified predicted a multifunctional protein rather than one strictly acting as an ion channel [343]. Also, monomeric NMR structural analysis performed on Flag-p7 from G1b identified a relevant allosteric cavity compatible with drug binding. These findings highlight the importance of p7 as a potential target for the development of HCV inhibitors [344].

NMR data obtained on oligomerized p7 indicated that the protein forms a hexameric channel complex in the presence of detergent, and EM analysis identified a flower-like shape with six distinctive petals with the p7 helices oriented toward the lumen of the ER. The identified structure also showed an accessible area facing the interior of the ER that could provide an interaction face with other viral or host proteins [345]. Additionally, the G1b p7 sequence can assemble sequentially to form an oligomerized structure of four to seven subunits with the most putatively functional oligomer being the hexamer in which the cylindrical structure accommodates the flower-like shape. The sequential formation of the channel was suggested to regulate the function of p7, whereby p7 would be retained in an inactive state that allows p7 to bind to other viral proteins early in the assembly process. Subsequently, pore formation could take place to accommodate channel activity required at a later stage in the life cycle [346]. A more recent NMR structural identification of the p7 ion channel showed that the G5a sequence forms an unusual hexameric complex with a funnel-like shape. This study also suggested that amantadine and rimantadine bind to six equivalent hydrophobic pockets between the pore-forming and peripheral helices, defining a possible mechanism of inhibitory action [347].

The diversity of structural predictions for p7 based on NMR analyses may be attributed to a number of variables, such as the different genotypic p7 amino acid

sequences, the purification method used, or the solvent and environmental conditions used in the preparation of the protein for NMR structural analyses. However, structural agreement in NMR studies will prove to be important for identification of structural features of monomeric and oligomeric p7 structures, as well as drug binding determinants within p7.

#### **1.10.4 Functional analyses of p7**

In 2003, deletion or mutations at the two conserved positively charged residues in the cytoplasmic loop of p7 within infectious clones of G1a failed to cause viremia after intrahepatic transfection in the chimpanzee model. Furthermore, substitution of G1a p7 with p7 from an infectious G2a clone was also not viable. These results clearly demonstrated the importance of p7 for the virus life cycle *in vivo*, as well as the restrictive genotypic context of p7 function [348]. Given the accumulating evidence suggesting that p7 forms an ion channel and acts as a viroporin, it is conceivable that such an activity might be required for the virus to facilitate entry, assembly or release in a manner similar to that of other viroporins [308,316,318].

After the development of the HCVcc system, mutagenesis studies on various viral proteins for functional analysis became possible [324-326]. Early mutational analyses demonstrated the importance of p7 for virus production [300,349]. Steinmann *et al.* showed that p7 is involved at a late stage of the replication cycle, since mutants in p7 reduced both total infectivity and the ratio of intracellular to released infectious particles. Successively, p7 was shown to be less likely involved in the viral entry process due to the maintenance of specific infectivity observed in released virions from genomes mutated in p7 [349]. A subsequent study illustrated that p7 mutant genomes generated lower levels

of intracellular virus, released core protein and infectious particles in the culture supernatant, suggesting involvement of p7 at an early stage of the viral morphogenesis cycle [300].

Currently, a number of convincing pieces of evidence exist suggesting that p7 is not involved in the entry steps of the viral life cycle: (i) HCV pseudoparticles (HCVpp) produced from 293T cells lack p7 but remain infectious to hepatocyte-derived cell lines [350]; The pseudoparticle system (HCVpp) exploited retrovirus biology to create retroviral particles with HCV glycoproteins expressed on the surface. 293T cells were used for generation of particles expressing E1 and E2, these particles could then be used to infect permissive hepatocytes and entry could be measured through the use of a luciferase or GFP reporter genes in the recombinant construct [350,351] (ii) cis-expression of p7 during HCVpp production did not increase infectivity [352]; (iii) HCV virions are acid-resistant, suggesting that ion channel activity during entry is not needed [131]; (iv) p7 from the related virus BVDV was not found to be a component of the virion structure [293], and (v) more importantly, using an infectious construct containing a double HA-tagged p7, it was found that p7 was not contained within the virion, even in concentrated, affinity-purified or Flag-tagged preparations of virus [306]. All things considered, a viroporin-like activity of p7 might be required at a step downstream of virus entry that resembles the influenza A M2 activity during viral egress and HIV-1 Vpu (see Table 1.2).

Regarding steps subsequent to viral entry, current evidence indicates that p7 has no role in RNA replication. Firstly, replicons lacking p7 replicate efficiently in Huh-7 cells [353,354], secondly BVDV containing mutations in p7 was able to replicate in cell

culture, but failed to produce infectious particles [307], and lastly mutations or deletions within p7 generated in the context of HCVcc replicate RNA efficiently with impaired infectious virus production [300,349].

Since the establishment of the HCVcc system, HCV assembly has been under intense investigation. One intriguing aspect of the assembly mechanism is the accumulation of core protein around a cellular organelle termed the LD, which is proposed to function as a platform for virus assembly [96]. However, others have suggested that core does not accumulate on LD in the context of an efficiently-assembling virus, suggesting that core/LD accumulation is an indication of an assembly defect [355]. It is worth mentioning that an NS2-mutated, assembly-deficient HCV strain could be rescued by compensatory mutations in p7 that restored virus production and colocalization of NS2, E2 and NS3 to a site in close proximity to LDs [65]. Likewise, another report visualized that p7 can manipulate the intracellular distribution of NS2 and disrupt its binding pattern with other viral proteins [172]. This assumption was confirmed recently by showing that NS2 distribution is affected by the presence of p7 and its N-terminal signal peptide [356]. Presenting a different perspective, the efficiency of core accumulation on LDs due to p7 mutations was quantified and it was found that mutations in the p7 cytoplasmic loop affected the unloading of core protein from LDs, resulting in a retention of core around LDs [30]. The preceding observations could indicate that p7 is important for colocalization of NS2 with LDs, a process that is crucial for NS2 function. Interestingly, p7 has been also shown important for unloading of newly-formed capsids toward further assembly, maturation and envelopment [30,357].

Subsequent to core protein recruitment to LDs, core oligomerizes to form capsid structures. This presumably facilitates the formation of multi-order forms during the morphogenesis cycle that would include monomeric core, partial oligomers, fully oligomerized capsids and virions associated with triglycerides and  $\beta$ -lipoproteins, all of which would show different densities upon intracellular lysate fractionation [41,358,359]. A study has shown that mutated p7 at the cytoplasmic loop did not prevent the assembly of core-containing intracellular particles after iodixanol equilibrium gradient sedimentation [360]. Compellingly, another report performed core quantitation on the fractions obtained after sucrose-based rate zonal fractionation and observed an increased proportion of incompletely assembled capsids. Further analyses on these fractions indicated that p7 mutations or deletions in the cytoplasmic loop led to accumulation of non-enveloped capsids in transfected cells [30]. These data reflect the necessity of p7 downstream of capsid formation.

The postulate that p7 can function as an ion channel gained momentum when it was shown that HCV infection equilibrates intracellular acidic vesicles. This loss of acidity was not observed with HCV genomes harboring p7 ion channel defective mutations (KR33/35AA in the J4 sequence or RR33/35AA in JFH-1). It was found that intracellular HCV virions were acid-sensitive, whereas extracellular virions were acid-stable. In confirmation of this finding, the acidification inhibitor Bafilomycin A1 was able to rescue virus production of ion channel defective mutants in Huh-7.5 cells [178]. Furthermore, HCV replication-defective viruses containing mutations in TM1 of p7 or the cytoplasmic loop showed a restoration of infectivity by *in trans* expression of the M2 proton channel of influenza A, indicative of an ion channel requirement for HCV propagation in cell

culture [360]. Interestingly, proteinase K digestion of p7 mutant viruses showed significant reductions in core protein, indicative of nucleocapsids lacking envelopes, which demonstrated that p7 affects virus production at a step prior to envelopment [30]. p7 ion channel activity could be an important step to protect the immature virion from an acidic environment during the late stage of assembly, particularly at the envelopment step.

In summation, therefore, it seems that p7 function may be two-fold: protection of virion-associated E2 from acid-induced degradation that is mediated by the ion channel activity; in the second role, p7 is required for proper targeting of viral glycoproteins through a concerted effort with NS2. A third potential function, which has received little attention to date, is that p7 may play a role in an interaction with a cellular factor. In this regard, p7 has been suggested to modulate cell death signaling and may be targeted by cellular kinases [361,362]. It would be interesting to know if p7 interacts with cellular proteins in order to promote virus production.

#### **1.10.5 p7 interactions with other viral proteins**

The binding pattern of p7 with other viral proteins is continually being revealed through genetic-based investigations and protein-protein interaction analyses. The first clue concerning the necessity for p7 to interact with other proteins came through intrahepatic RNA injection of chimpanzees with a chimeric virus generated by replacing the p7 sequence from G1a with that of G2a. The resulting chimera was not viable after injection, illustrating p7's genotype-specific interactions [348]. The JFH-1 infectious clone was used in multiple studies to generate infectious chimeras by replacing the core-NS2 (or in some cases core to part of NS2) fragment of the JFH-1 sequence (G2a) with



that of other genotypes. In this case it was observed that chimeric viruses were only viable when there was genotypic homology between p7 and the substituted structural genes. This characteristic of chimera construction suggested there is an important interplay between p7 and one or more structural proteins [23,60,363]. To successfully create a viable chimeric construct, H77 (G1a) was fused to JFH-1 (2a) at a site within NS2 and passaged to allow accumulation of adaptive/compensatory mutations. The resulting chimera acquired mutations within p7, NS2, E1 and NS3. The combination of p7 and NS2 mutations increased specific infectivity, suggesting an interaction between p7 and NS2. This interaction was proposed to anchor p7 to NS2 within the membrane, thereby enhancing the ability of p7 to protect the nascent virion from premature inactivation [363]. Furthermore, compensatory mutations in p7 (F776L/S) significantly enhanced the fitness of defective core mutants in the context of a J6/JFH-1 chimera, providing genetic evidence for an interaction between p7 and core [364]. Taken together, it remains unlikely that a protein the size of p7 interacts with multiple viral proteins. That being said, it is possible that p7 interacts with a single viral protein in order to stabilize other downstream interactions and promote viral propagation.

Biochemical and proteomic assays have also been used to identify p7 binding partners. One report that utilized tagged NS2 showed efficient pull-down with p7, E2, NS3 and to a lesser extent, NS5A [65]. Other work has shown NS2 can be co-immunoprecipitated with E1 as well [172]. Importantly, mutagenesis studies have demonstrated that p7 affects NS2 colocalization with E2 and NS3 around LDs, and p7 basic loop mutations significantly reduced NS2 interactions with E1, E2 and NS3 [65,172]. It is noteworthy that mutations in the two basic residues of the cytoplasmic loop

of p7 caused considerable alteration of the intracellular distribution of NS2 and E2 [172]. However, it is difficult to conclude whether these results indicate a direct interaction with the loop, or if the removal of the charged residues changed the topology of p7 to indirectly affect the localization of other proteins. Recently, NS2 was confirmed to interact with p7 and E2 as mutations in p7 changed subcellular localization of NS2 and reduced viral assembly [357]. p7 interaction with NS2 was shown to be independent of p7 ion channel activity and NS2 localization was affected primarily by p7 and its signal peptide at the N-terminus, suggesting an alternative function of p7 during the assembly process [356]. More recently, a co-immunoprecipitation study using a replication-competent virus containing a double HA-tagged p7 confirmed the specific interaction between p7 and NS2, which was proposed to be critical for virus production in cell culture [306]. Despite the discrepancies observed for NS2 as a p7 binding partner we can conclude that NS2 maintains the organization of the viral assembly process and that p7 is a mandatory interaction partner. It is also plausible that p7 binds NS2 in a manner that regulates its function in virus assembly.

The size and topology of p7 has made it poorly immunogenic for antibody production. Consequently, the determination of p7 binding partners by standard methods is problematic and difficult to interpret. The topology of p7 in membrane structures and data generated from purified or tagged proteins might not reflect the actual picture. Considering p7 exists as multiple forms: unprocessed E2-p7, p7-NS2, monomeric p7 or oligomers, reproducibility of data becomes further complicated. Accordingly, more accurate experimental approaches need to be investigated or identified to better define the protein-protein interaction activities of p7, both viral and potentially cellular.

### 1.11 Project design and hypothesis

The only functional study of p7 *in vivo* indicated that p7 is essential for successful intrahepatic infection in the chimpanzee model and illustrated its critical role in the viral life cycle [348]. Studies employing full-length HCVcc chimeras have indicated that p7 is also important in virus production and acts at a late stage during viral assembly and/or release [300,349,363]. It has also been shown that p7 interacts with NS2 and that this interaction is required for efficient viral assembly and release [65,172,306,355,357]. In addition, p7 was shown to impact cellular organelle pH and reduce the number of highly acidic vesicles more rapidly than ion channel defective p7 mutant sequences [178]. Recently, another group has shown that p7 is important for formation of completely assembled capsids and enveloped virions, as p7-defective HCV genomes showed different sedimentation profiles on density gradients and generated proteinase K sensitive virions; a possible indication of envelope deficiency [30]. However, it is still unknown at which step p7 acts during the HCV life cycle. The impact of p7 regulation of intracellular vesicles and interaction with NS2 on virus production remain important questions. The aim of this study was to further elucidate the role of p7 in the HCV life cycle. We hypothesized that p7 is involved in the HCV assembly process at a late stage after nucleocapsid formation.

The first part of this project was to test the effect of p7 in infectious virus production using the HCVcc system. A number of studies have already performed mutational analyses on p7 and reported the importance of this protein for infectious virus production both *in vivo* and *in vitro* using the chimpanzee model and HCVcc system, respectively [300,348,349,363]. However, these reports focused mostly on the two basic residues

within the cytoplasmic loop since it was shown to be important for *in vitro* ion channel activity and intracellular vesicle pH modulation. In our study, and for the first time, we have created a panel of triple alanine mutations covering most of TM1, the cytoplasmic loop and TM2 of p7 in order to pinpoint which domains and amino acids are important in p7 function. We believe that a more comprehensive mutational analysis might provide additional clues into p7 function.

The adapted strain of JFH-1, termed JFH1<sub>D</sub>, was used for the majority of the experiments outlined in this work. The reason behind using the JFH1<sub>D</sub> strain in this report was because it replicates with high efficiency compared to wild-type JFH-1 [365]. Due to the high replication rate of JFH1<sub>D</sub> it would be less expected to acquire adaptive mutation during passage that could potentially confound the effect of our mutation of interest. However, to prevent even JFH1<sub>D</sub> from acquiring such confounding mutations, experiments addressing virus production and assembly were confirmed in S29 cells, which represent a single-cycle virus production assay. S29 cells are a sub-clone of Huh7 that express significantly lower levels of CD81 (an HCV entry receptor), and therefore, permit RNA replication and virus production, but do not support entry. Using this system provides the ability to analyze infectious virus production without the confounding effect of multiple rounds of infection cycles, during which mutant reversion could happen [365].

The second part of this project was to investigate at which step of the viral life cycle p7 plays its role. There still exists a debate on the precise role of p7 in the HCV life cycle. However, multiple studies have shown that p7 acts at a late stage during viral assembly and/or release [300,349]. The observed interaction of p7 with the viral protein NS2 has been suggested to be required for efficient viral assembly and/or release

[65,172,355,357]. p7 was shown to reduce the number of intracellular acidic vesicles more than defective p7 mutants at the two basic residues of the cytoplasmic loop, emphasizing the importance of p7 role in protecting HCV particles from acidic inactivation [178]. Similarly, transfection of HCV genomes defective at the cytoplasmic loop of p7 was shown to generate proteinase-K sensitive virions, a possible indication of envelope deficiency [30]. In spite of this, the exact role of p7 is still unclear. How the previously identified interaction pattern of p7 with other viral proteins and the viroporin activity contributes to the whole HCV life cycle still remains to be elucidated. The aim of this part was to identify at which step of the virus life cycle p7 affects virus production. Detailed functional characterization of the effects of generated mutations at multiple stages of the HCV life cycle were performed, including polyprotein processing, core/LD association, core protein oligomerization and virus release.

The last part of this study was aimed at performing forced evolution studies in which the mutants with defects in virus production were cultured for a prolonged period of time so that they would have the opportunity to acquire compensatory mutations that would correct for the effects of the originally-engineered mutations. Such compensatory mutations are key to providing clues as to which domains of other proteins might form critical interactions with p7. Forced evolution studies on HIV provided important information in respect to assembly and viral genome incorporation [366-368]. When this approach was performed in full-length and chimeric HCV genomes, it led to the generation of multiple adaptive strains of HCV that are currently used instead of the low replicating wild-type JFH-1 strain [365,369]. This strategy was also performed on genomes containing mutations in viral proteins other than p7 and provided information

regarding the interaction patterns of such proteins [359,363]. This is the first study to utilise this approach on HCV genomes harboring p7 mutations. The subsequent analysis we performed on one of the compensatory mutations we identified provides strong clues regarding p7 function.

### **1.12 Research objectives**

- i. To determine the effect of HCV p7 on production of infectious virus (chapter 3).
- ii. To identify the most important domains/amino acids residues of p7 for virus propagation in cell culture (chapter 3 and 4).
- iii. To investigate at which step of the virus life cycle p7 plays its role (chapter 4).
- iv. To determine potential viral proteins that bind to p7 using the forced evolution analysis (chapter 5).

## CHAPTER 2: MATERIALS AND METHODS

### 2.1 Cell culture

Transfections and infections were performed in Huh-7.5 cells (generous gift from C.M. Rice, Rockefeller University/Apath Inc. LLC, USA [353]) and S29 cells (subclone of Huh-7 cells representing a single-cycle virus production assay [365], generous gift from S. Emerson, NIH, USA). Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen/Life Technology) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin, referred to as complete medium (CM). All cells were grown at 37°C in 5% CO<sub>2</sub>.

### 2.2 Cloning and plasmid construction

Plasmids were constructed mainly in the backbone of JFH1<sub>D</sub>, which was itself derived from a cell culture-adapted JFH-1 strain (genotype 2a genome previously described, see section 2.11). Other adapted strains of JFH-1 backgrounds were also used and illustrated in Figure 2.1. PCR mutagenesis was employed to introduce the desired mutations with primers that included the *Bsi*WI and *Not*I restriction sites within the JFH1<sub>D</sub> backbone (primers listed in Table 2.1). The JFH1<sub>D</sub> plasmid and PCR products were digested with *Bsi*WI and *Not*I (New England Biolabs) and fragments gel-purified then ligated using the Rapid DNA Ligation Kit (Roche). The ΔGDD negative control was created using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) and specific primers that omitted the GDD motif of NS5B. The same strategy was also employed to create other plasmid backgrounds including: (i) the JFH1<sub>A4S</sub> backbone was created by using a primer encoding the E1 A4 epitope in the backbone of JFH1<sub>S</sub>, which includes

only the NS2 adaptive mutation, and (ii) the JFH1<sub>D-FLAG</sub> background that was created by transferring the gene segment between *Bsi*WI and *Ale*I restriction sites from a FLAG-modified JFH-AM2 that was a gift from T. Wang (University of Pittsburgh, USA) [370]. Selected p7 mutations were then recreated by substituting the *Bsi*WI and *Ale*I digested fragment from the original p7 mutant plasmids into JFH1<sub>A4S</sub> and JFH1<sub>D-FLAG</sub>. All plasmid sequences were verified by enzymatic digestion and double-stranded DNA sequencing.

### **2.3 *In vitro* transcription and RNA transfection**

Mutant and control plasmids were linearized by *Xba*I digestion for 2 h at 37°C and 1 µg of each linearized plasmid was used for *in vitro* RNA transcription using the T7 Megascript kit (Ambion). RNA integrity was verified by gel electrophoresis. RNA transcripts were transfected using DMRIE-C reagent (Invitrogen/Life Technology) into 1x10<sup>6</sup> Huh-7.5 or S29 cells plated in 10 cm cell culture dishes 24 h prior to transfection. We preferred to use DMRIE-C over electroporation because it provides comparable transfection efficiencies, but more importantly, it shows no cytotoxicity effects and transfection can be done easily in case of adherent cells such as those used in this project (Huh7.5 and S29).

### **2.4 Antibodies**

The following antibodies were used in this study: mouse anti-HCV core monoclonal antibody (mAb) (B2; Anogen) was used for both indirect IF at a dilution of 1:200 and Western blot (WB) analysis at a dilution of 1:1000; mouse anti-NS3 mAb at a dilution of 1:5000 (C65371M; Meridian Life Science); mouse mAb A4 (anti-E1; a kind gift from Harry Greenberg, Stanford university, USA); mouse anti-NS2 (6H6, a kind gift from Charles M. Rice, Rockefeller university, USA) both used for WB analysis at dilutions of



1:2000; mouse anti-E2 (AP33; generous gift from Genentech) used at a dilution of 1:1000; mouse anti-GAPDH mAb at a dilution of 1:10000 (Abcam); sheep anti-NS5A antiserum (a kind gift from Mark Harris, University of Leeds, UK) used for WB analysis at dilutions of 1:10000. HCV core in gradient analyses was detected using mouse anti-core mAb (MA1-080; Pierce Research) at a dilution of 1:1000, and mouse anti-ADRP (Progen Biotechnik) was used at a dilution of 1:1000. Alexa Flour® 488 anti-mouse secondary antibody (Invitrogen/Life Technology) was used for indirect IF at a dilution of 1:1000. Goat anti-mouse and donkey anti-sheep horseradish peroxidase (HRP)-labelled secondary antibodies (Santa Cruz Biotechnology) were used for WB analysis at a dilution of 1:1000.

## **2.5 Indirect IF**

Cells were grown on 8-well chamber slides (Thermo Scientific), fixed in 100% acetone for 2 min, washed with phosphate buffer saline (PBS) pH 7.4 and incubated with primary antibody for 20 min. Then, slides were washed 3 times with PBS and incubated with the secondary antibody for an additional 20 min and washed 3 times with PBS. Slides were then mounted with Vectashield Hard Set mounting medium containing DAPI (Vector Laboratories).

## **2.6 Virus titration**

Virus titres were determined by endpoint dilution assay for focus-forming units (ffu) as described previously [325]. Briefly, 8-well chamber slides were seeded with  $4 \times 10^5$  Huh-7.5 cells per well 24 h prior to infection. At 72 h post-transfection, cell supernatants were clarified through Millex-HV 45 µm filters (Millipore) before being serially diluted 10-fold with complete DMEM. 100 µl of each dilution was inoculated for 4 h before

being removed and replaced with complete DMEM. At 72 h post-infection cells were fixed with acetone and core protein was visualized by indirect IF. Virus titres were expressed as the number of ffu per ml of supernatant, where a focus was defined by a cluster of 3 or more infected cells.

## **2.7 Titration of intracellular infectious virus**

At 72 h post-transfection, cells were trypsinized, pelleted by centrifugation at 400 x g and re-suspended in 1 ml of CM. The re-suspended cells were then lysed by 4 cycles of freeze/thaw (3 min freeze/ 3 min thaw) in a dry ice/methanol bath and pelleted by centrifugation at 1500 x g. Virus titres were determined as described above.

## **2.8 SDS-PAGE and WB analysis**

At indicated time-points post-transfection, cells were trypsinized, pelleted by centrifugation at 400 x g and re-suspended in 300 µl of passive lysis buffer (Promega). Cellular debris was pelleted by centrifugation after 30 min incubation on ice. A fraction of the cell lysates was then loaded in a 1:1 ratio with 2X loading dye for SDS-PAGE. To visualize extracellular core protein, at 72 h post-transfection 12 ml of transfection supernatant (pooled from 2x10 cm dishes) was passed through a 0.45 µm filter and subjected to ultracentrifugation at 80,000 x g for 4 h at 4°C using a Sorvall TH-614 rotor. Pellets were re-suspended in 20 µl of 2 X loading dye and the full amount was subjected to SDS-PAGE and WB. All WBs performed in this study were exposed and analyzed using the luminescent image analyzer (Image Quant LAS 4000, GE Santé Bio-Sciences).

## **2.9 Confocal microscopy**

At 72 h post-transfection, cells were seeded onto 8-well chamber slides and 48 h later washed with PBS and fixed with 4% paraformaldehyde for 20 min, then washed and

permeabilized with 0.1 % Triton X-100 for 15 min. Following this, cells were washed and incubated with anti-core Ab at a 1:200 dilution in 5% BSA/PBS. Next, cells were washed 3 times and incubated with anti-mouse labelled with Alexa Fluor® 488. To visualize LD the HCS LipidTOX Deep Red neutral lipid stain (Invitrogen/Life Technology) was added to Vectashield Hard Set mounting medium containing DAPI (Vector Laboratories) at a 1:200 dilution then added to the slides and examined by laser scanning confocal microscopy.

### **2.10 Iodixanol density gradient fractionation**

Two 10 cm plates per construct were transfected as described above and cells were trypsinized 72 h post-transfection, pelleted by centrifugation and re-suspended in 0.5 ml of lysis buffer (50 mM Tris (pH 7.5), 140 mM NaCl, 5 mM EDTA and 0.5% Triton-X100) and incubated for 30 min on ice then cleared by centrifugation. The clarified 0.5 ml were loaded over 4.5 ml of pre-formed 10-50% iodixanol gradients (prepared using OptiPrep Density Gradient Medium (Sigma) and Hank's balanced salt solution (Invitrogen/Life Technology)). Samples were then ultracentrifuged at 100,000  $\times g$  for 16 h at 4°C using a Beckman SW55Ti rotor. Next, ten fractions (0.5 ml/fraction) for each construct were collected from the top of each tube. 50  $\mu$ l from each fraction was retained before the protein precipitation process for measurement of density of each fraction using a refractometer (Fisher Scientific). Proteins were extracted from the remainder of each fraction by methanol precipitation at -20°C for 40 min, and then pelleted by centrifugation. The resultant pellets were re-suspended in 50  $\mu$ l of 2 X loading buffer, boiled and then probed for core protein by WB analysis.

The same exact procedure was also performed by re-suspending the pellet from two plates for each mutant and control with 0.5 ml CM and subjecting them to multiple cycles of freeze/thaw instead of using the above mentioned lysis buffer to compare core sedimentation profiles using two different lysis methods (freeze/thaw *vs.* chemical lysis with detergent).

### **2.11 Bafilomycin A1 and ammonium chloride treatments**

4 pM Bafilomycin A1 (dissolved in DMSO) and 12 mM  $\text{NH}_4\text{Cl}$  were prepared and used to treat Huh-7.5 or S29 cells that had received mutant or control RNA 48 h previously. These concentrations were chosen after testing a range of 1–50 pM (Bafilomycin A1) and 6–50 mM (ammonium chloride ( $\text{NH}_4\text{Cl}$ )). Supernatants from harvested cells were used for titer determination or WB as outlined previously.

### **2.12 Forced evolution assay**

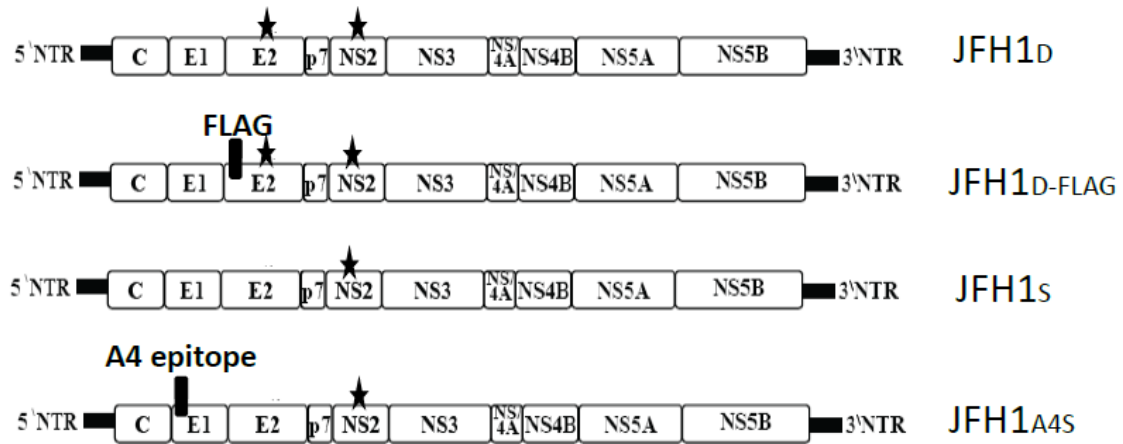
For each control and mutant, cells were washed and trypsinized 72 h post-transfection and re-suspended in 10 ml CM. 2 ml of suspension were then combined with 7 ml CM. 200  $\mu\text{l}$  of suspension combined with 200  $\mu\text{l}$  CM for each mutant and control were seeded on 8-well chamber slides and incubated for 48 h at 37°C to assess viral spread as an initial indication of virus adaptation. The same procedure was repeated for up to 10 rounds or until a significant increase was observed in core staining on the chamber slide after each round. Cells were also monitored daily for any visual cell death and in such a case, the passaging was terminated. A fraction of clarified supernatants from each mutant was saved from each round and used to confirm virus adaptation using the infection titration assay.

### **2.13 Reverse transcription, PCR amplification and sequencing of rescued mutants**

To identify compensatory mutations that had arisen during passage of the mutant(s), supernatant from the passage of interest plate was first used for 2 rounds of infection to increase the rescued virus population. RNA was extracted from supernatants using TRIzolLS (Invitrogen). Reverse transcription was performed to generate cDNA from the adaptive mutants. Multiple PCR reactions using primers designed to cover the whole HCV genome were then performed, as previously described [365,371]. Sequencing of the PCR products was performed at the Centre for Applied Genomics at the Hospital for Sick Children (Toronto, Canada).

### **2.14 Flow cytometric analysis of intracellular core protein**

S29 cells transfected with the mutants or controls were collected after trypsinization and washed 4 times with PBS. Cells pellets were then prepared for staining using FIX & PERM® Cell Fixation and Permeabilization Kits (Invirogen) following manufacturer's instruction. After being washed with PBS, cells were incubated with antibody to HCV core (1:400 dilution) for 30 min followed by 3 washes and incubation with FITC-labeled secondary antibody at a 1:1000 dilution for 30 min, then washed 3 times. Pellets were resuspended in 500 µl PBS and analyzed by flow cytometry.



**Figure 2.1 Illustrative diagram of the different strains of JFH-1 used in this study.** The adaptive mutations indicated by the stars are as follow: the E2 adaptive mutation is N417S and the NS2 adaptive mutation is Q1012R. FLAG and A4 epitope locations are also indicated. The strain names are shown on the right side of the panel.

**Table 2.1 Primers used to generate p7 mutations**

<b>Mutation name</b>	<b>Background</b>	<b>Primer sequence</b>
p7 (1)	JFH1 <sub>D</sub> , JFH1 <sub>S</sub> , JFH1 <sub>A4S</sub> & JFH1 <sub>D-FLAG</sub>	F-CTGCGAGTGCGGCT <b>GCCGCGAGCT</b> TGGCCTCCTATATTTTGC R-GCATCGCGTGGGCCGTCACTATAT
p7 (2)	JFH1 <sub>D</sub> JFH1 <sub>D-FLAG</sub>	F-GCGGCTAACTGCCATG <b>CCGCCGC</b> ATATTTTGCCATCTTC R-GCATCGCGTGGGCCGTCACTATAT
p7 (3)	JFH1 <sub>D</sub> , JFH1 <sub>A4S</sub> JFH1 <sub>D-FLAG</sub>	F-TGCCATGGCCTCCTAG <b>CTGCTGTC</b> ATCTTCTTCGTGGC R-GCATCGCGTGGGCCGTCACTATAT
p7 (4)	JFH1 <sub>D</sub> , JFH1 <sub>A4S</sub> & JFH1 <sub>D-FLAG</sub>	CTCCTATATTTTGGC <b>GCTGCCGC</b> AGTGGCAGCTTGGCAC R-GCATCGCGTGGGCCGTCACTATAT
p7 (5)	JFH1 <sub>D</sub> JFH1 <sub>D-FLAG</sub>	F-TTTGCCATCTTCTTC <b>GCGGTAGT</b> TTTGGCACATCAGGGG R-GCATCGCGTGGGCCGTCACTATAT
p7 (6)	JFH1 <sub>D</sub> , JFH1 <sub>A4S</sub> & JFH1 <sub>D-FLAG</sub>	F-TTCTTCGTGGCAGCT <b>GCTGCCGCC</b> CAGGGGTGGGTCC R-GCATCGCGTGGGCCGTCACTATAT
p7 (7)	JFH1 <sub>D</sub> , JFH1 <sub>A4S</sub> & JFH1 <sub>D-FLAG</sub>	F-GCAGCTTGGCACATC <b>GCGGCTGC</b> AGTGGTCCCCCTTGACC R-GCATCGCGTGGGCCGTCACTATAT
p7 (8)	JFH1 <sub>D</sub>	F-GGGTCGGGTGGTC <b>GCCGCCGCC</b> ACCTATTGCCTCA
p7 (9)	JFH1 <sub>D</sub>	F-GGTCCCCTTGACC <b>GCCGCTGCC</b> CTACTGGCCTAT
p7 (10)	JFH1 <sub>D</sub>	F-GACCACCTATTG <b>CCGCGCAG</b> CCCTATGGCCCTTCT
p7 (11)	JFH1 <sub>D</sub>	F-TTGCCCTCACTGGC <b>GCGCGC</b> CTTCTGCCTACTGC
p7 (12)	JFH1 <sub>D</sub>	F-TGGCCTATGGCCC <b>GCCGCCGC</b> ACTGCTCATGGCAC
p7 (13)	JFH1 <sub>D</sub>	F-GCCCTTCTGCCTAG <b>GCCGCCGCC</b> GGCACTGCCCCGGC
N765D in p7 (1)	p7 (1) plasmid	F-GCTGCGAGTGCGGCT <b>GACG</b> CAGCTGGCCTCCTATATTTTGC
N765D in p7 (2)	p7 (2) plasmid	F-GCGGCT <b>GACT</b> GCCATGCCGCCGCATATTTTGCCATCTTC
N765D in p7 (3)	p7 (3) plasmid	F-GCTGCGAGTGCGGCT <b>GACT</b> GCCATGGCCTCCTAGCTGCTGTC
N765D in p7 (4 to 13) *	p7 (4) to p7 (13) plasmids	F-GCTGCGAGTGCGGCT <b>GACT</b> GCCATGGCCTCCTATATTTTGC
JFH1 <sub>A4S</sub>	JFH1 <sub>S</sub>	F-GGTGAAGAAT <b>TCCAGTGGCCT</b> TACC <b>CAC</b> GTGACCAATGAC

List of the primers used to generate p7 mutations. Bold letters represent the changes introduced on the indicated backgrounds to introduce the desired mutations. Where the reverse primer is not provided means the reverse complement sequence of the forward primer was used as reverse primer.

\*Same primers were used to accommodate N765D in the backgrounds of p7 mutations from p7(3) to p7(13) using their respective background created previously.

## **CHAPTER 3: RESULTS (EFFECT OF p7 ON INFECTIOUS VIRUS PRODUCTION)**

Results in this chapter along with those in chapter 4 have been published in *Virus Research*, 2013; 176(1-2):199-210.

### **3.1 Generation of p7 mutations**

Previous reports have identified adaptive and compensatory mutations within p7 that enhanced virus production [363,365,372-374]. The cytoplasmic loop region contains highly conserved basic residues, K33 and R35, known to impact infectivity in chimpanzees, as well as *in vitro* ion channel activity and virus production in cell culture [300,303,348,349]. Based on these findings we hypothesized that a comprehensive mutational analysis of TM1 and the cytoplasmic loop might provide additional insight into the function of this protein. Accordingly, seven alanine triplet mutations (termed p7(1) to p7(7)) were generated to cover the TM1, TM2 and cytoplasmic loop of p7 (Figure 3.1). These mutations were constructed in the background of a full-length, cell culture-adapted strain of HCV JFH-1 termed JFH1<sub>D</sub>, which contains adaptive mutations in E2 (N417S) and NS2 (Q1012R) that function cooperatively to increase infectious virus production [365].

### **3.2 Effect of p7 mutations within TM1 and the cytoplasmic loop on infectious virus production using single-cycle assay**

To test the effects of the generated mutations on infectious virus production, we used a single-cycle virus production assay. Here, the mutant RNA genomes were transfected into S29 cells, which express very low levels of CD81, the major receptor for HCV entry



[365]. These cells permit RNA replication and virus particle production, yet do not support virus entry, allowing a comparison between mutants and their ability to generate infectious progeny without the confounding effects associated with multiple infectious cycles. Core, NS3 and NS5A WB analyses performed on transfected S29 cell lysates at 72 h post-transfection showed similar band intensities among the mutants when compared with JFH1<sub>D</sub>. This indicated that transfection efficiencies were comparable and protein expression/processing was not affected by the mutations generated in p7 (Figure 3.2A). These comparable protein levels also exclude possible effects on RNA replication or genome instability that may have been caused by the generated mutations. The negative control ( $\Delta$ GDD) contained a deletion within the active site of the HCV NS5B polymerase and, therefore, produced no core, NS3 or NS5A proteins.

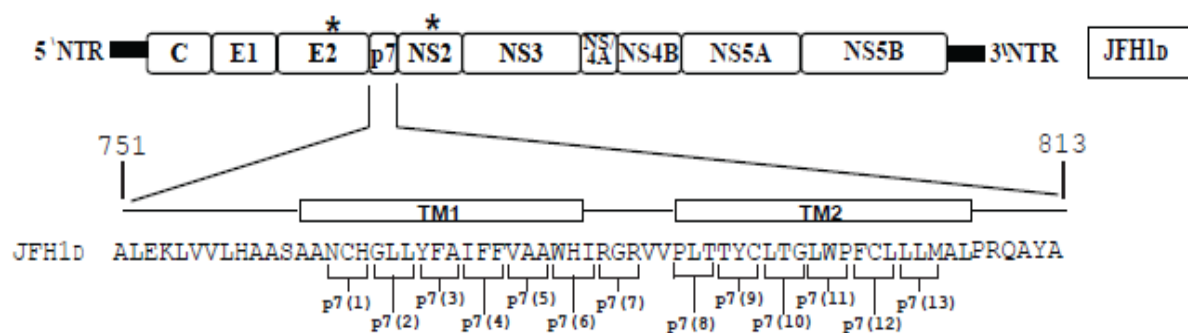
At 72 h post-transfection, S29 cells were trypsinized and re-plated in 8-well chamber slides. Two days later, indirect IF analysis confirmed that all mutant genomes and the JFH1<sub>D</sub> control possessed relatively similar HCV core staining patterns, with approximately 25% of cells being positive for core protein (Figure 3.2B). Cells transfected with  $\Delta$ GDD displayed no core-positive cells.

Next, to quantify the effects of the generated mutations on virus production, we measured the levels of infectious virus present in the supernatants of transfected cells (Figure 3.2C). Mutations p7(1) (located at the N-terminal end of TM1) and p7(4) (located near the C-terminal end), as well as mutations p7(6) and p7(7) (within the cytoplasmic loop) reduced infectious virus production to levels lower than the detection limit of the assay. In contrast, p7(2) and p7(3), located in the central region of TM1, decreased virus production by ~2 logs. The mutation near the carboxy-terminal end of TM1 (p7(5))

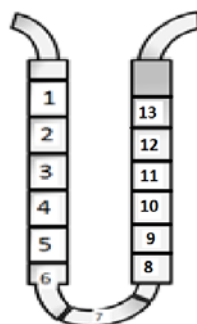
showed little effect on virus production, which was likely due to the conservative amino acid changes comprising this mutation (VAA-AVV). Taken together, these data indicated that TM1 and the cytoplasmic loop of p7 are important for production of infectious virus, and that the amino acids located within the central region of TM1 are seemingly less critical for p7 function.

### **3.3 Effect of TM1 and the cytoplasmic loop mutations on infectious virus production using Huh-7.5 cells**

To verify the single cycle assay findings, we repeated the above experiments in Huh-7.5 cells and found that the levels of core, NS3 and NS5A proteins on WBs were apparently different among the mutant viruses (Figure 3.3A). Core IF staining patterns were also different from that observed in S29 cells (Figure 3.3B), demonstrating that mutations p7(1), p7(4), p7(6) and p7(7) substantially affected virus spread. Mutant virus p7(5) exhibited similar core staining to that of JFH1<sub>D</sub> with >90% of the cells positive for core. The  $\Delta$ GDD control displayed no core signal. Virus production at 72 h post-transfection of Huh-7.5 (Figure 3.3C) was higher than that from S29 cells (Figure 3.3C), but overall patterns between mutants were similar (minor differences between cell lines for p7(1) and p7(6) were disregarded since they fell under the assay cut-off). The observed differences on protein levels, core staining patterns and virus titers correlated with the effects of virus spread and are, therefore, due to the amplification and accumulation of virus that occurs in the permissive Huh-7.5 cells, which is in agreement with previous findings [359]. These results indicated that the generated mutant genomes are indeed defective in infectious virus production.

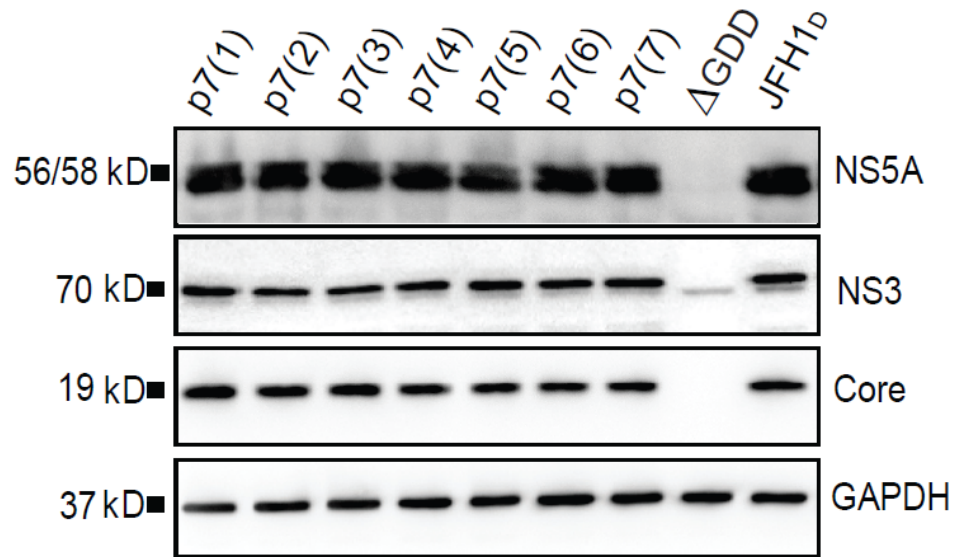


Mutation Name		p7(1)	p7(2)	p7(3)	p7(4)	p7(5)	p7(6)	p7(7)	p7(8)	p7(9)	p7(10)	p7(11)	p7(12)	p7(13)
Mutated Residues		765-767	768-770	771-773	774-776	777-779	780-782	783-785	788-790	791-793	794-796	797-799	800-802	803-804
AA Change	From	NCH	GLL	YFA	IFF	VAA	WHI	RGR	PLT	TYC	LTG	LWP	FCL	LLM
	To	AAA	AAA	AAA	AAA	AVV	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA

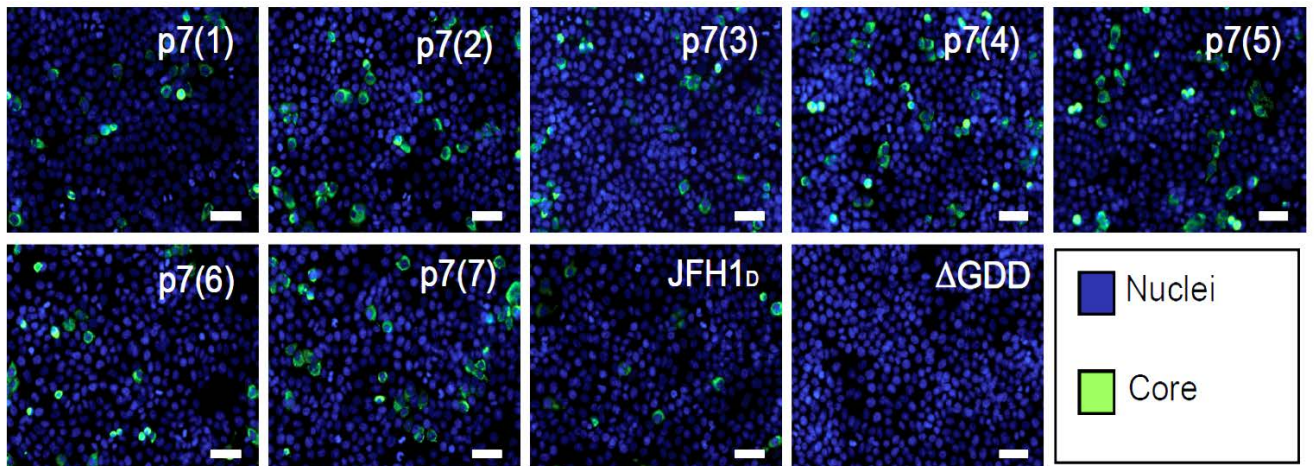


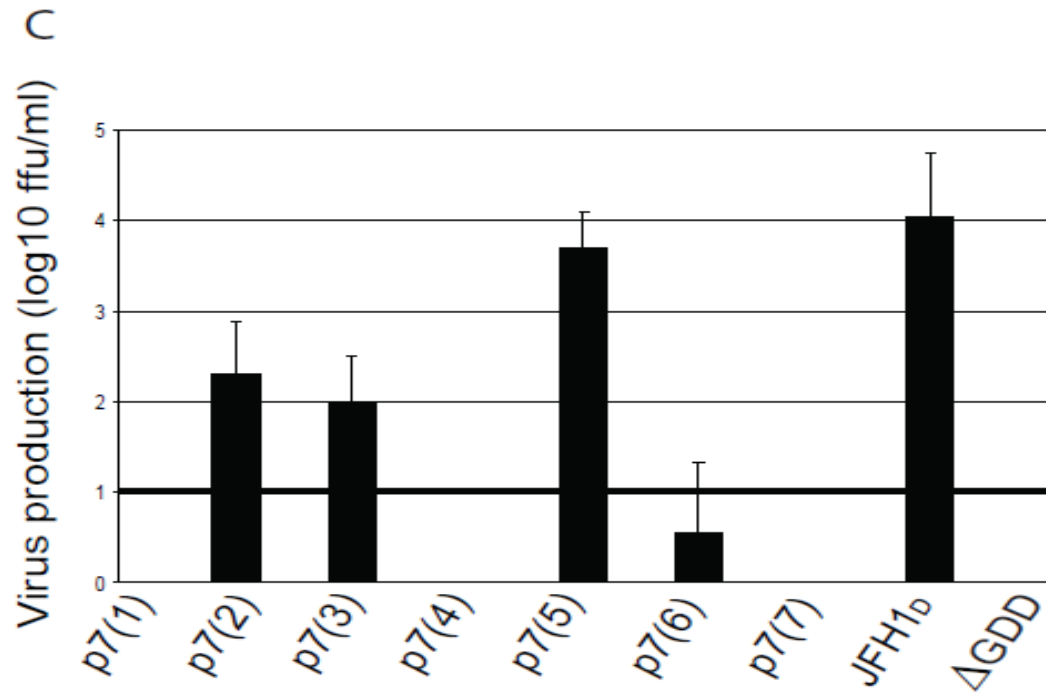
**Figure 3.1 Construction of p7 mutants.** 13 alanine triplet mutants were constructed in the background of JFH1<sub>D</sub>, which exhibits enhanced infectious virus production compared to JFH-1 due to the presence of adaptive mutations in E2 (N417S) and NS2 (Q1012R), (indicated by \*). The p7 TM1, TM2 and the cytoplasmic loop with the amino acid compositions are shown, with mutated residues, and affected polyprotein amino acids numbers are shown in the table. Valine was introduced at positions where the original amino acid was alanine. The lower diagram indicates the locations of the p7 mutants on the membrane topology of p7.

A



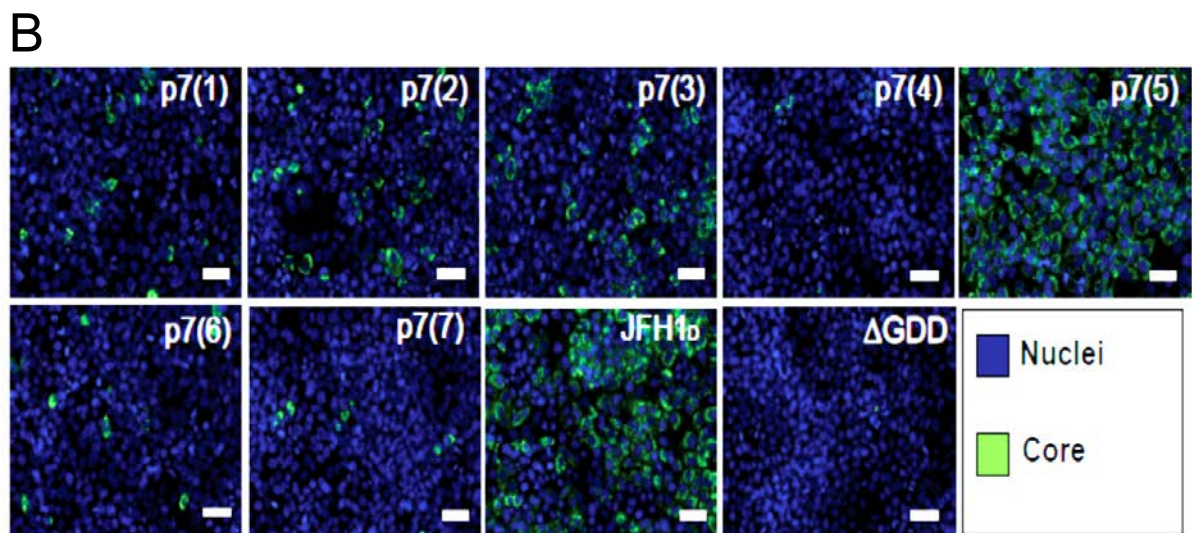
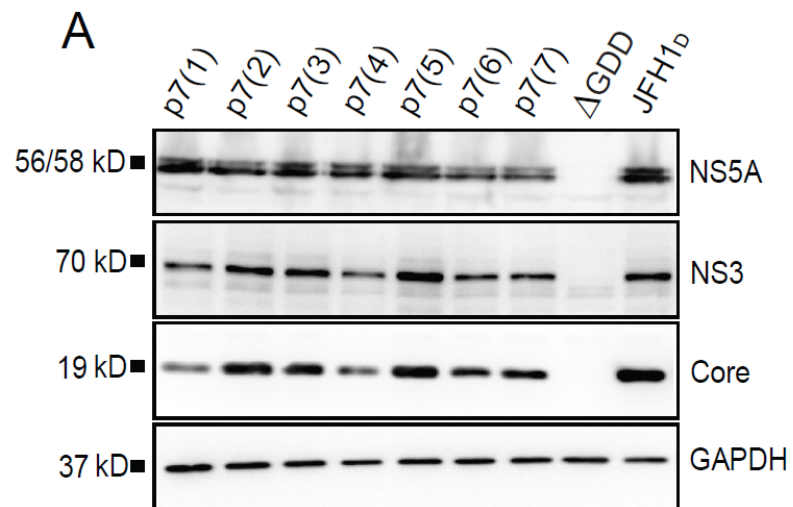
B

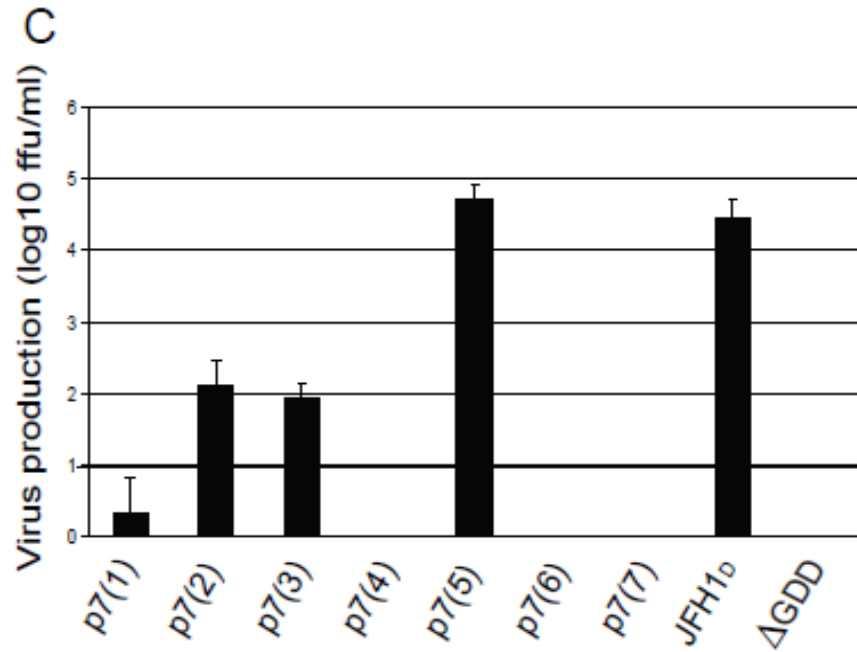




**Figure 3.2 Single cycle virus production assay analysis of p7 mutant viruses. (A)**

S29 cells transfected with the p7 mutants or controls (JFH1<sub>D</sub> and ΔGDD) were lysed and probed with antibodies recognizing core, NS3, NS5A and GAPDH by WB analysis. **(B)** S29 cells were transfected with equivalent amounts of transcribed RNA representing p7 mutants and controls (JFH1<sub>D</sub> and ΔGDD). At 72 h post-transfection, cells were seeded onto 8-well chamber slides and two days later washed, fixed and stained for core (green) and DAPI (blue). Scale bars represent 50 μm. **(C)** Culture supernatants were filtered and serially diluted to infect naïve Huh-7.5 cells. At three days post-infection, virus titers were determined by limiting dilution focus-forming assay. Focus-forming assays were performed in triplicate and error bars represent standard error of the mean. The bold line represents the cut off of the assay, which was 10 ffu/ml. Results represent three independent transfection/infection experiments.





**Figure 3.3 The p7 cytoplasmic loop and TM1 are important for virus production.**

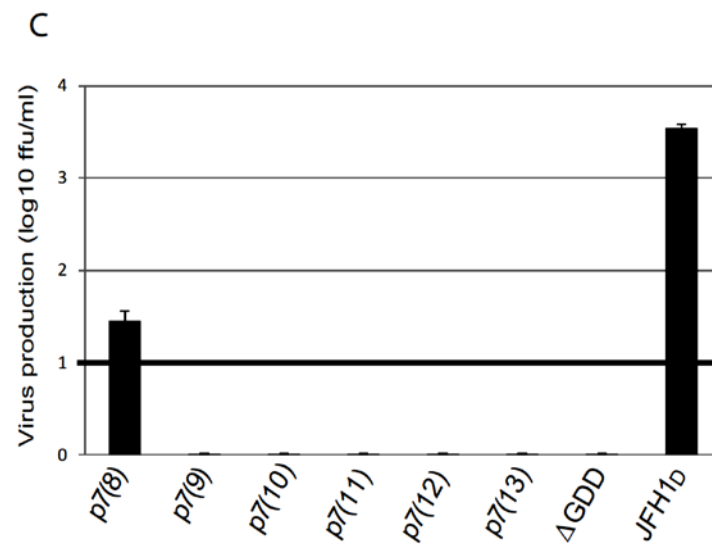
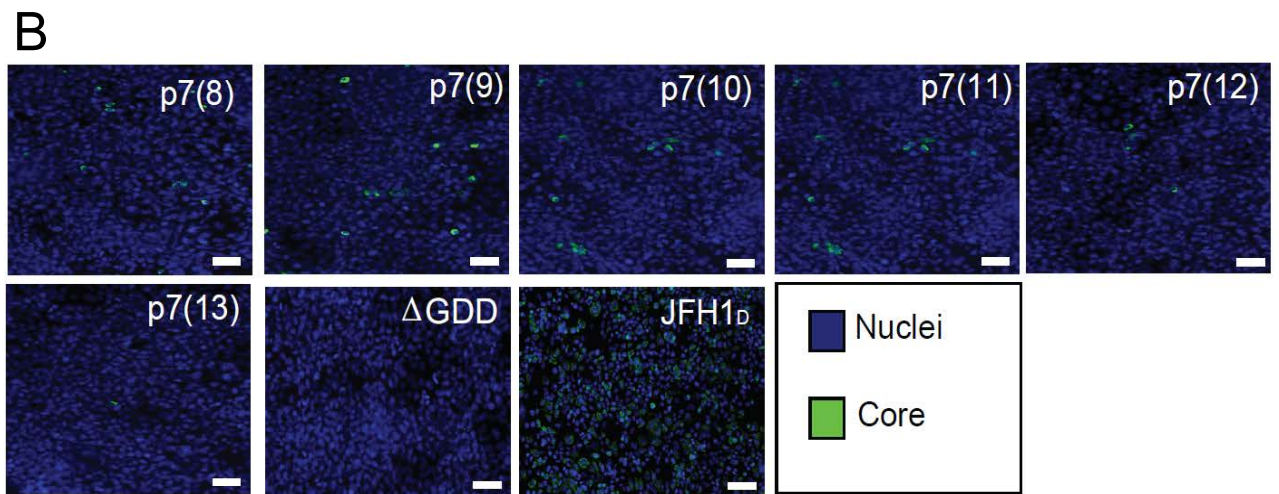
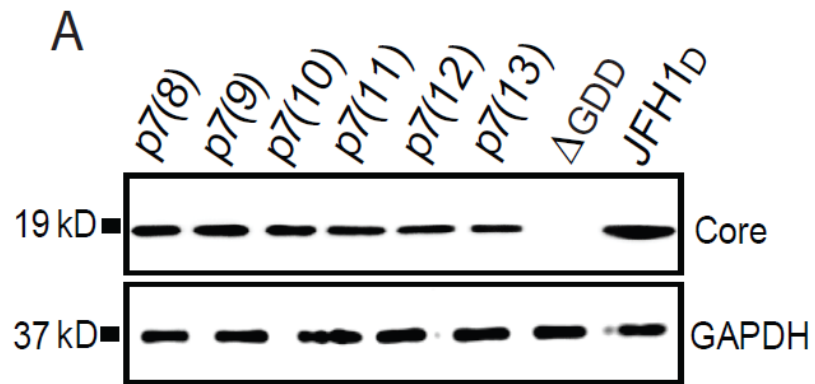
(A) Huh-7.5 cells transfected with the p7 mutant panel and controls were lysed and probed with antibodies recognizing core, NS3, NS5A and GAPDH by WB. Results are representative of three independent experiments. (B) Huh-7.5 cells were transfected with equivalent amounts of transcribed RNA representing p7 mutants and controls (JFH1<sub>D</sub> and ΔGDD). At 72 h post-transfection cells were seeded onto 8-well chamber slides and two days later washed, fixed and stained for core (green) and DAPI (blue). Scale bars represent 50 μm. (C) Culture supernatants were filtered and virus titers determined in Huh-7.5 cells. Titrations were performed in triplicate and error bars represent standard error of the mean.

### **3.4 Effect of TM2 mutations on infectious virus production using Huh-7.5 cells**

In this study a set of triple alanine changes covering TM2 was also constructed, attempting to expand our knowledge on the functionally essential portions of the p7 protein. Huh-7.5 cells were transfected with the generated constructs and controls. 72 h later cells were trypsinized and subjected to WB analysis for core (Figure 3.4A). Levels of the core protein were different among mutants. The negative control  $\Delta$ GDD produced no detectable level of core, as expected. The core protein levels correlated with the effects on virus spread (Figure 3.4B) after an IF staining of core protein on the constructs and control done at 72h post-transfection. Therefore, these results indicated that the generated mutants did not affect genome stability or polyprotein processing.

Consequently, to quantify the effects of the generated mutations on infectious virus production, clarified supernatants from each mutation and control at 72 h post-transfection were used to infect naïve Huh-7.5 cells followed by a ffu assay (Figure 3.4C). We found that TM2 of p7 is also important for virus production in cell culture with a maximum effect appearing with p7(9 to 13) causing a decrease in titres to below detectable levels. While mutation p7(8), located at the C-terminus of TM2, showed a lesser defect on virus production than the rest of TM2 mutations, it maintained a significant reduction of approximately 2 log less than the control JFH1<sub>D</sub>. Taken together, these results suggest that all domains of p7 are critical for infectious virus production. We did not examine the expression of other viral proteins or confirm infectious virus production using the single-cycle assay for the TM2 mutations because these mutations were only to be further used in forced evolution assays (see chapter 5).





**Figure 3.4 The p7 TM2 is important for virus production.** (A) Huh-7.5 cells transfected with the p7 mutants (8-13) and controls were lysed and probed with antibodies recognizing core and GAPDH by WB. Results are representative of two independent experiments. (B) Huh-7.5 cells were transfected with equivalent amounts of transcribed RNA representing p7 mutants and controls (JFH1<sub>D</sub> and  $\Delta$ GDD). At 72 h post-transfection cells were seeded onto 8-well chamber slides and two days later washed, fixed and stained for core (green) and DAPI (blue), and visualized under 20 X magnification. (C) Culture supernatants were filtered and virus titers determined in Huh-7.5 cells. Titrations were performed in triplicate and error bars represent standard error of the mean.

## **CHAPTER 4: RESULTS (ANALYSIS OF THE ROLE OF p7 IN THE HCV LIFE CYCLE)**

Results in this chapter along with those in chapter 3 have been published in *Virus Research*, 2013; 176(1-2):199-210. Only the mutations at TM1 and the cytoplasmic loop were analyzed in this section.

### **4.1 Analysis of intracellular and extracellular species of virus particles**

The reduction of extracellular infectious virus exhibited by some of the p7 mutants could result from two possibilities: (i) p7 mutants exhibit wild-type levels of released virus particles, but these have reduced infectivity, or (ii) an overall reduction in the production of particles. To determine which of these possibilities occurred, supernatants from Huh-7.5 cells transfected with the mutants and controls were collected, clarified, layered over a 20% sucrose cushion and subjected to ultracentrifugation. Huh-7.5 cells were used in this case to maximize yields of virus. WB analysis was then performed on the pelleted material (Figure 4.1A). Core protein was detectable only for genomes that produced high levels of infectious particles (JFH1<sub>D</sub> and p7(5)), demonstrating that the mutations introduced into p7 decreased the production of core-containing particles, as opposed to reducing the infectivity of secreted virions. The mutations p7(2) and p7(3) were shown to produce low levels of infectious virus, but particles could not be detected in the supernatant as this assay was not sensitive enough to detect such small amounts of virus production.

From the results outlined above, we next investigated whether the p7 mutants that produced little or no extracellular infectivity were also compromised for intracellular infectivity. Accordingly, S29 cells transfected with p7 mutants and controls were

subjected to multiple freeze/thaw cycles to obtain intracellular infectious virus at 72 h post-transfection. In parallel, filtered supernatants were collected and viruses from both sources were used to inoculate naïve Huh-7.5 cells to measure infectious titers (Figure 4.1B). All mutants with reduced extracellular titers also displayed similar reductions in the levels of intracellular infectious virus. Taken together, these results demonstrate that the mutations made within p7 resulted in an absence of core-containing HCV particles outside the cell, most likely resulting from their compromised ability to generate infectious particles in the intracellular environment.

#### **4.2 Mutations in p7 TM1 and the cytoplasmic loop do not affect core sedimentation profiles**

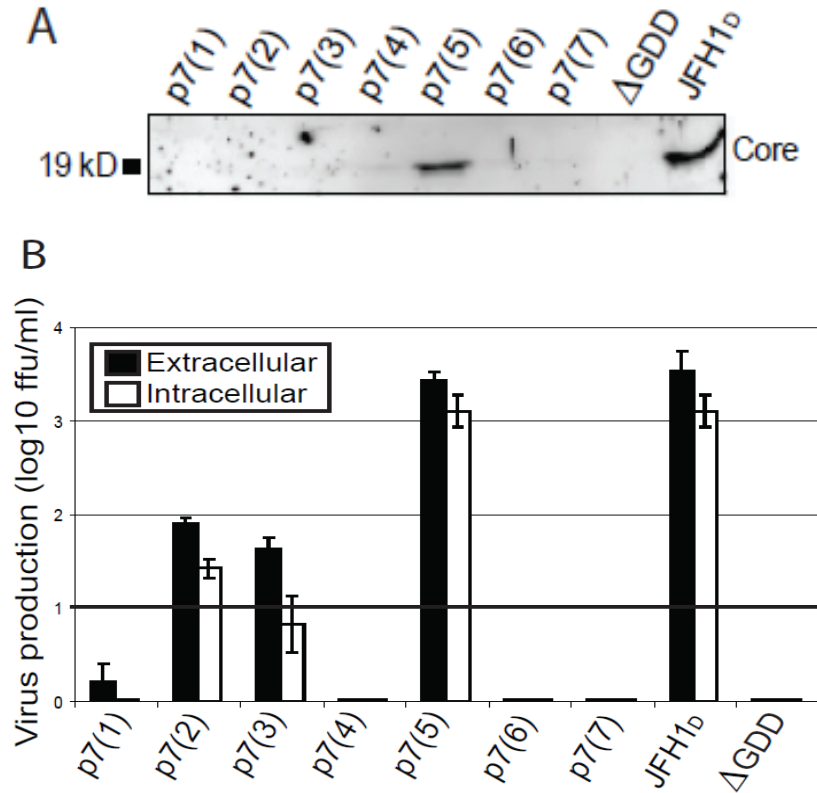
The above data indicated that the selected p7 mutations within TM1 and the cytoplasmic loop render HCV unable to produce intracellular infectious virions. However, it was unclear whether these mutants were assembling intracellular particles that were non-infectious, or were incapable of building the nucleocapsid structure. Typically, density gradient fractionation of transfected cell lysates has revealed that intracellular HCV core exists at various densities, presumably representing sequential stages of capsid assembly. These species are thought to include monomeric core associated with LD, and oligomerized core representing both newly forming nucleocapsids and virions associated with triglycerides and  $\beta$ -lipoprotein (VLDL and LDL) complexes [41,358,359,375]. Therefore, we sought to determine whether any of our p7 mutants displayed altered patterns of core distribution following centrifugation through density gradients. To do this, two mutations within TM1 (p7(2) and p7(4)) and one mutation within the cytoplasmic loop (p7(7)) were selected for gradient analysis

because they spanned the TM1 and cytoplasmic loop region of p7. Also, these mutants displayed a spectrum of effects on virus production; (p7(2)) showed a moderate reduction, whereas p7(4) and p7(7) produced no detectable infectious virus. These mutant RNA genomes were transfected into Huh-7.5 cells, and 72 h later, intracellular lysates were harvested using TNE buffer containing 0.5% Triton X-100. This mild detergent was chosen to preserve core-core oligomers within the lysate while disrupting core-membrane complexes that may otherwise be sufficiently dense to traverse into the gradient and be misinterpreted as multi-ordered structures. Lysates were then loaded over 10-50% iodixanol gradients and subjected to ultracentrifugation. Ten gradient fractions were collected from top (fraction 1) to bottom (fraction 10) and analysed by WB for core (Figure 4.2). In all constructs tested, varying core protein levels were observed in fractions 1-3, likely representing monomeric core species (densities ranging from 1.019 to 1.090 g/cm<sup>3</sup>). The strongest core bands, which we propose represent naked viral nucleocapsids, typically appeared in fractions 6 and 7 with a density range of 1.145-1.169 g/cm<sup>3</sup>. It is unlikely that these core structures were enveloped/complexed with membranes, since no infectivity was associated with these or any fractions. While slight variations were regularly observed in these assays, we did not observe any consistently noticeable differences between JFH1<sub>D</sub> and any of the p7 mutants tested with respect to core patterns on WB, except that JFH1<sub>D</sub> showed higher core band intensities in the blots due to its ability to spread and produce more infectious virus. However, using a lysis buffer containing a detergent capable of breaking core-core interactions (TNE + 0.5% SDS) resulted in a loss of core from fractions 6 and 7, with most of the protein now being found in the top fractions of the gradient (Figure 4.2). It should be noted that a

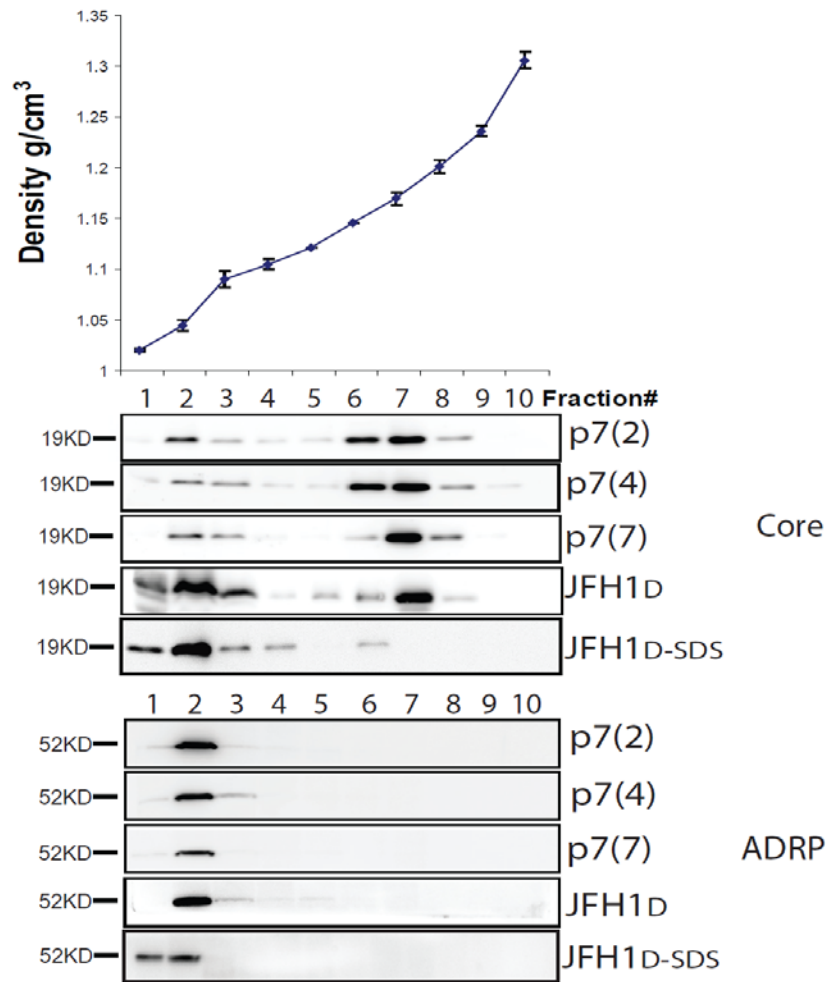
comparison of lysis methods (freeze-thaw *vs.* TNE+0.5% TX-100) on cells harbouring JFH1<sub>D</sub> revealed differences in core banding patterns, indicating that these distinct lysis methods influence the migration of liberated core through iodixanol gradients (Figure 4.3). Similarly, spinning harvested intracellular particles through a sucrose cushion prior to their ultracentrifugation through an iodixanol gradient made little difference (with the exception of filtering out core in fractions 1-3) to those results obtained without a cushion (Figure 4.3). In comparison, we also observed no differences between p7 mutants and JFH1<sub>D</sub> in core and viral RNA sedimentation profile when lysates were prepared by freeze-thaw (Figure 4.4). Of further note is that RNA quantitation for core-containing gradient fractions was irrelevant, since gradients run on a negative control ( $\Delta$ GDD) also gave a peak of RNA in these fractions (Figure 4.4). This final result suggests that gradient-derived RNA patterns obtained from intracellular lysates are complicated by the existence of input RNA delivered during the transfection process. Overall, the data obtained from density gradient experiments suggest that the p7 mutants tested here were competent for nucleocapsid assembly.

#### **4.4 p7 TM1 and the cytoplasmic loop do not affect core targeting to LD**

We next wished to determine whether p7 might influence the recruitment of core protein to LD, the proposed platform for virion formation [96]. Accordingly, Huh-7.5 cells were transfected with the mutant genomes and stained for core protein and LD. As shown in Figure 4.5, no differences in core/LD association were observed between the JFH1<sub>D</sub> control and any of the p7 mutants studied in this chapter. These data conclusively demonstrate that neither p7 TM1 nor the cytoplasmic loop affect the targeting of core protein to LD.

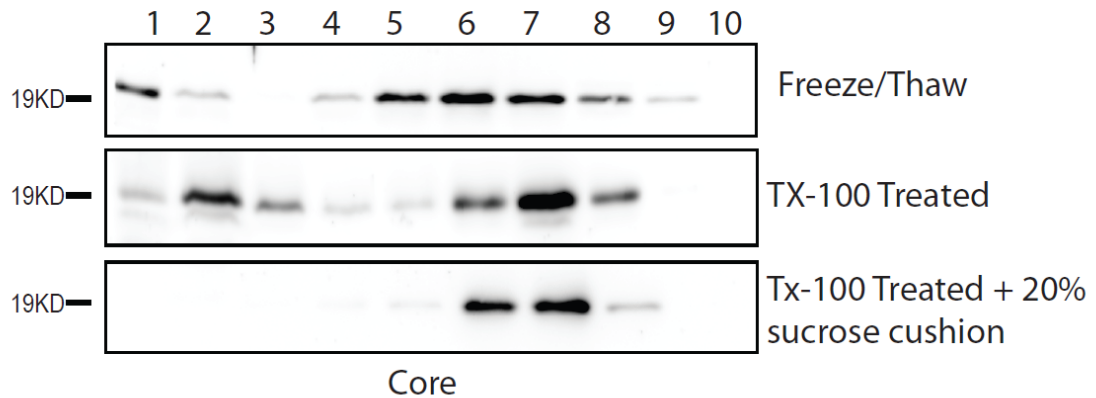


**Figure 4.1 Extracellular particle production and comparison of intracellular vs. extracellular infectious virus.** **(A)** Huh-7.5 cells were transfected with equivalent amounts of transcribed RNA representing p7 mutants and controls (JFH1<sub>D</sub> and ΔGDD) for 72 h. 12 ml of supernatants (pooled from 2x10 cm dishes) representing p7 mutants and controls were loaded over 2 ml of 20% sucrose, ultracentrifuged and the pellet probed by WB for core protein. Results are representative of two independent transfection/infection experiments. **(B)** S29 cells were transfected as above and 72 h post-transfection extracellular (solid bars) and intracellular (open bars) infectious virus production were measured by focus-forming assay in triplicate, and means plus standard errors are plotted. Results are representative of two independent transfection/infection experiments.

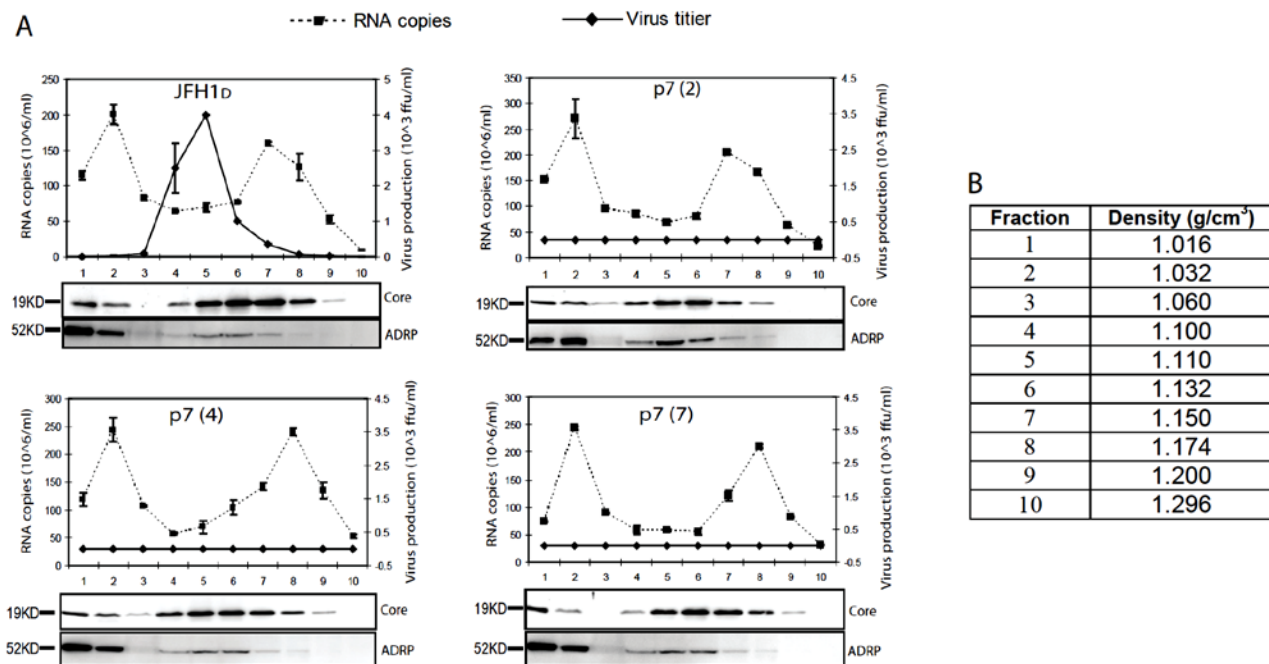


**Figure 4.2 Iodixanol gradient analysis of p7 mutant viruses using lysis buffer with detergent.** Huh-7.5 cells were transfected with RNA representing selected p7 mutant viruses including TM1 mutants p7(2) and p7(4) as well as the cytoplasmic loop mutant p7(7), and JFH1<sub>D</sub> was used as a control. At 72 h post-transfection intracellular lysates were harvested, loaded over preformed 10-50% iodixanol gradients and subjected to ultracentrifugation. Ten fractions from each tube were collected from top (fraction 1) to bottom (fraction 10). Density measurements (g/cm<sup>3</sup>) on each fraction were measured using a refractometer (top panel), and WB analyses for core and ADPR (LD marker) were performed (middle and bottom panels). Results are representative of three independent transfection/infection experiments.

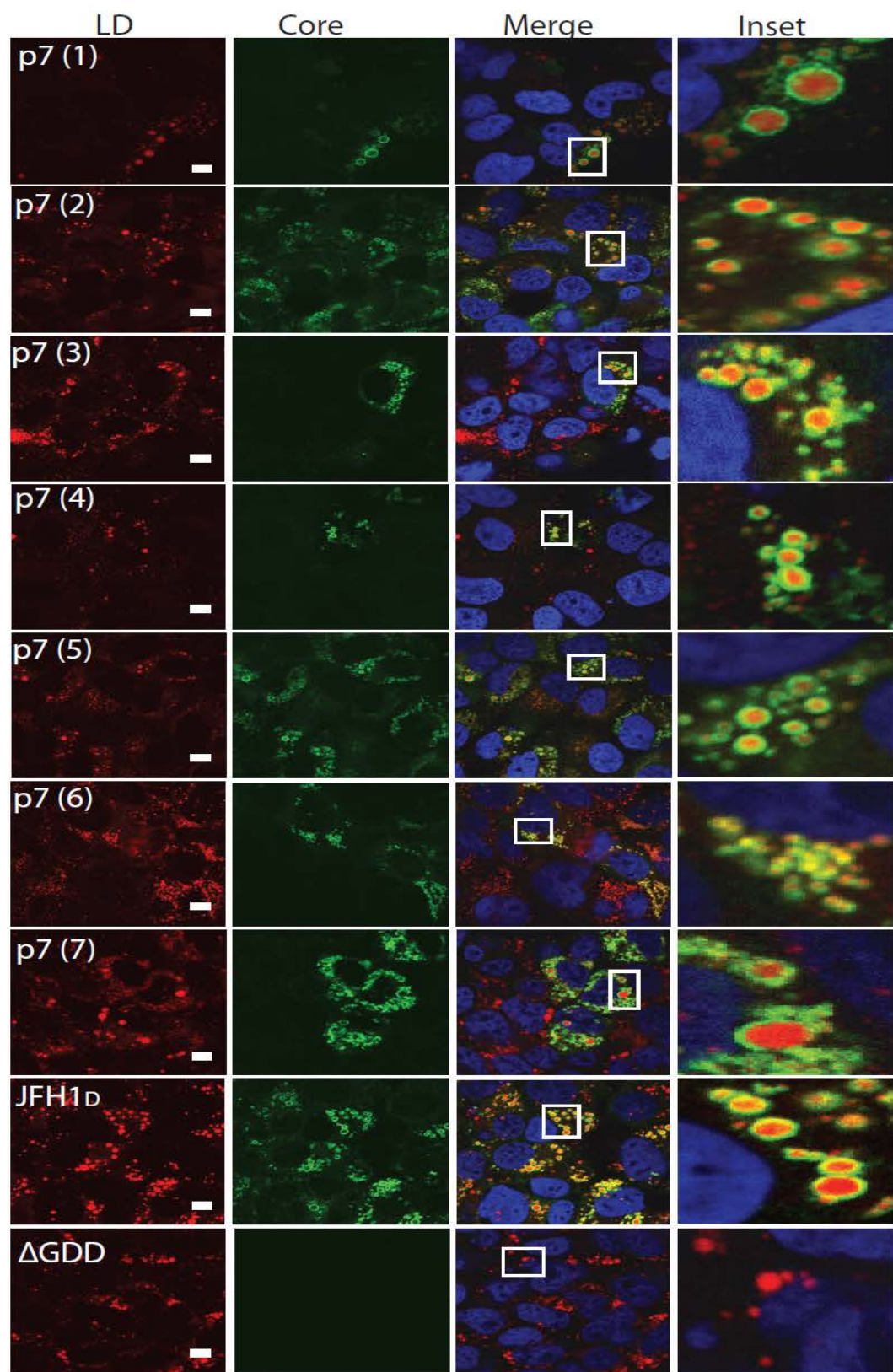




**Figure 4.3 Comparison of iodixanol gradient separation of core species, for JFH1<sub>D</sub> using different conditions.** Huh-7.5 cells were transfected with RNA encoding JFH1<sub>D</sub>. At 72 h post-transfection intracellular lysates were prepared by multiple freeze/thaw cycles (top panel), treated with TNE buffer containing 0.1% TX-100 (middle panel) or lysed with TNE containing TX-100 and sedimented through a 20% sucrose cushion (bottom panel). The resultant lysates were loaded over preformed 10–50% iodixanol gradients and subjected to ultracentrifugation. Ten fractions from each tube were collected from top (fraction 1) to bottom (fraction 10) and WB analyses for core were performed. Results are representative of three independent transfection experiments.



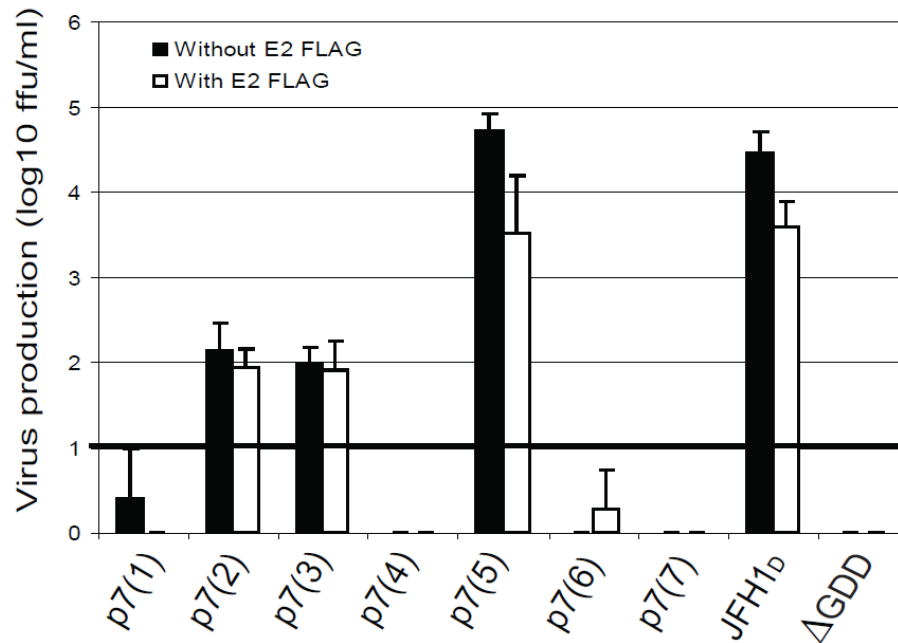
**Figure 4.4 Iodixanol gradient analyses of p7 mutant viruses after lysis using freeze/thaw method.** (A) Huh-7.5 cells were transfected with RNAs of the indicated p7 mutants and controls. At 72 h post-transfection intracellular lysates were obtained by multiple freeze and thaw cycles and loaded over preformed 10-50% iodixanol gradients. Ten fractions from each tube were collected from top (fraction 1) to bottom (fraction 10) and Real-Time RT-PCR (dashed line), virus titre (solid line), and WB analyses for core and ADRP were performed. (B) Density measurements (g/cm<sup>3</sup>) on each fraction were measured using a refractometer. Results are representative of three independent transfection/infection experiments.



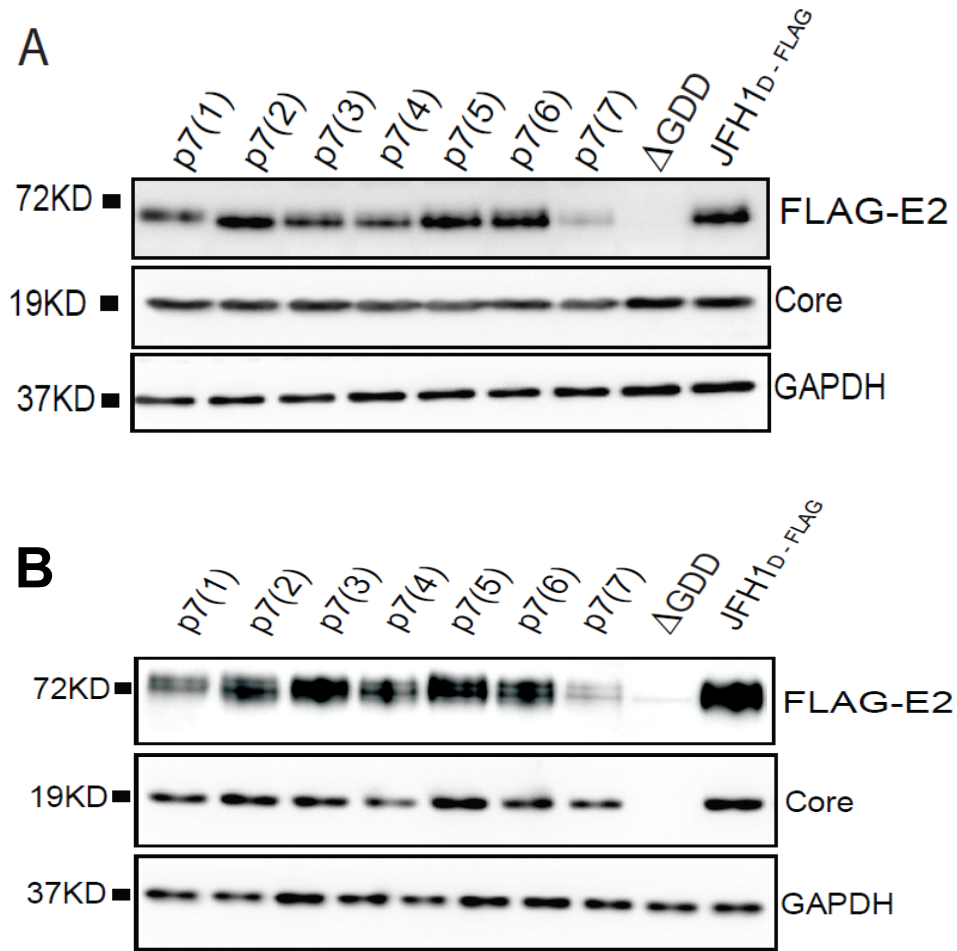
**Figure 4.5 Analysis of core/LD association.** Huh-7.5 cells were transfected with equivalent amounts of transcribed RNA representing p7 mutants and controls (JFH1<sub>D</sub> and  $\Delta$ GDD). At 72 h post-transfection, cells were seeded onto 8-well chamber slides and two days later washed and stained as described above. Cells were visualized by confocal microscopy under oil immersion. Scale bars represent 10  $\mu$ m. Enlarged areas from the merged image are shown on the right. Blue represents DAPI-stained nuclei, green fluorescence represents HCV core protein, and red represents LD staining. Results are representative of three independent experiments.

#### **4.5 Mutation of the p7 cytoplasmic loop results in a time-dependent reduction of E2 levels**

It has previously been shown that p7 can regulate pH in intracellular compartments [178], presumably to protect the virus from acid-induced degradation during egress. However, p7 may have an additional, separate function that involves an interaction with NS2, and this interaction may be important for localization of p7 with other viral proteins, including core and E2, at the site of assembly [65,172,306,355,357]. Therefore, it became essential to test some of our p7 mutants for an effect on viral glycoproteins E1 and E2. To do this, we recreated the panel of p7 mutations in a version of JFH1<sub>D</sub> that contained a substituted FLAG tag sequence within E2, termed JFH1<sub>D</sub>-FLAG. The use of the FLAG tag was necessary because the N417S adaptive mutation in E2 contained within the JFH1<sub>D</sub> backbone is located within the epitope recognized by most antibodies that recognize JFH-1 E2. To rule out the possibility that the FLAG sequence would cause confounding effects on virus production, we measured the amount of infectious virus produced from the FLAG-containing p7 mutant RNAs and observed a slight, but relatively insignificant, reduction in virus titre compared to that of without FLAG (Figure 4.6). Importantly however, the same pattern of effects on infectious virus production was observed for these mutants compared to their counterparts that lack the FLAG sequence. Upon transfection of S29 cells with these p7 mutants, WB analysis indicated that all mutants displayed similar levels of core protein (Figure 4.7A). However, this was not the case for E2, where some variation in E2 levels was observed among mutants, with the greatest reduction being apparent in p7(7) (cytoplasmic loop mutant containing the two conserved basic residues). These results were confirmed in Huh-7.5 cells (Figure 4.7B).



**Figure 4.6 p7 mutants in the background of JFH1<sub>D</sub>-FLAG showed a similar pattern of virus infectivity as JFH1<sub>D</sub> virus.** Huh-7.5 cells were transfected with equivalent amounts of RNA representing p7 mutants and controls (JFH1<sub>D</sub>, JFH1<sub>D</sub>-FLAG and ΔGDD). At 72 h post-transfection culture supernatants were clarified and virus titres determined. Assays were performed in triplicate and error bars represent standard error of the mean. Results are representative of two independent transfection/infection experiments.

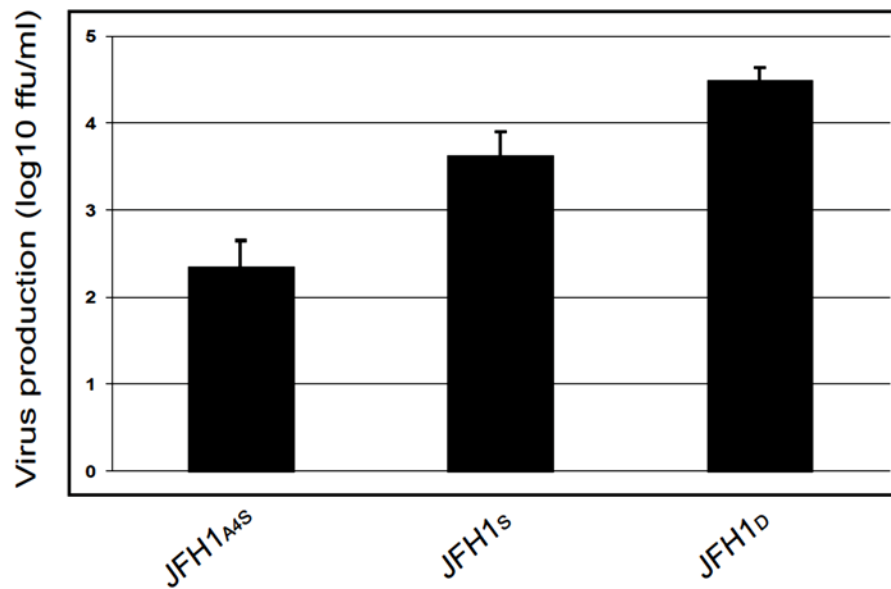


**Figure 4.7 Effects of p7 mutation on FLAG-E2 levels in S29 and Huh-7.5 cells.** S29 (A) and Huh-7.5 (B) cells were transfected with p7 mutants generated in the background of JFH1<sub>D</sub>-FLAG along with controls (JFH1<sub>D</sub>-FLAG and  $\Delta$ GDD). At 72 h post-transfection intracellular lysates were obtained and probed with antibodies against E2, core, and GAPDH by WB analysis.

To rule out the possibility that this effect on E2 was related to the presence of the FLAG sequence, we first created a new construct termed JFH1<sub>S</sub> that is the same as JFH1<sub>D</sub>, except that it lacks the N417S adaptive mutation in E2. This construct had to be used to allow detection of native E2 by antibodies that bind JFH-1 E2. In turn, JFH1<sub>S</sub> was then further modified by substituting a short sequence of E1 for the analogous sequence from HCV strain H77, to produce JFH1<sub>A4S</sub>. This strategy, reported elsewhere [66], renders E1 detectable by the E1 antibody A4. The modifications required to produce both these constructs did reduce virus production by approximately 15-fold (JFH1<sub>S</sub>) and 70-fold (JFH1<sub>A4S</sub>) compared to JFH1<sub>D</sub> (Figure 4.8). However, the level of virus production remained sufficient for analysis.

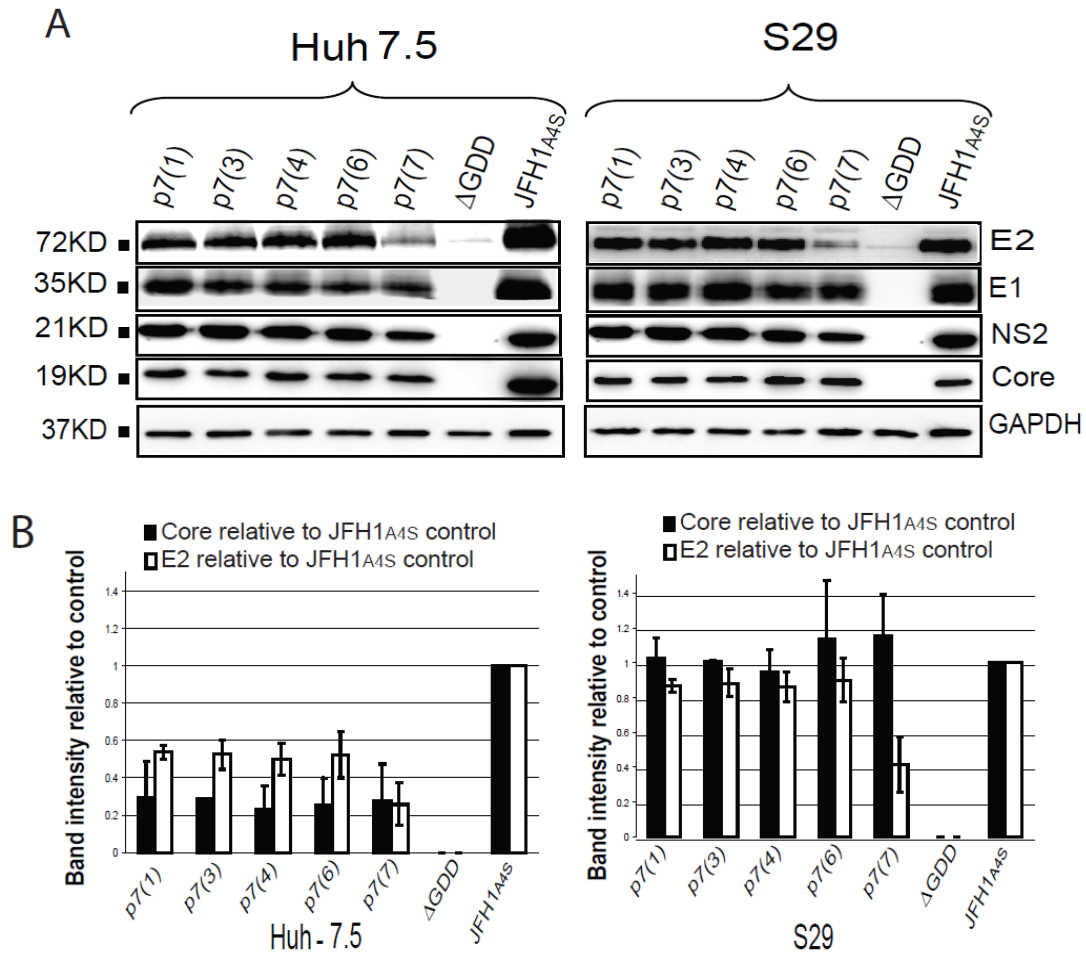
To examine effects on both E1 and E2, we first introduced selected p7 mutations (1, 3, 4, 6 and 7) into the background of JFH1<sub>A4S</sub>. WB analysis indicated that all mutants displayed insignificant variations in core, NS2 and E1 protein levels (Figure 4.9). However, this was not the case for p7(7) (the loop mutant containing the two conserved basic residues), where a substantial reduction in E2 was apparent. These results were confirmed in S29 cells (Figure 4.9). To analyze the reduction in E2 more quantitatively, the WB band intensities for E2 were measured by densitometry relative to that of core expressed from each mutant. Additionally, NS2 levels were measured in this manner (Figure 4.9). This analysis conclusively demonstrated that E2 levels expressed from p7(7) were notably lower compared to JFH1<sub>A4S</sub> and the other p7 mutants, whereas NS2 levels remained unchanged. These results suggest that modification of the dibasic residues within the p7 cytoplasmic loop leads to a reduction of E2 glycoprotein levels.





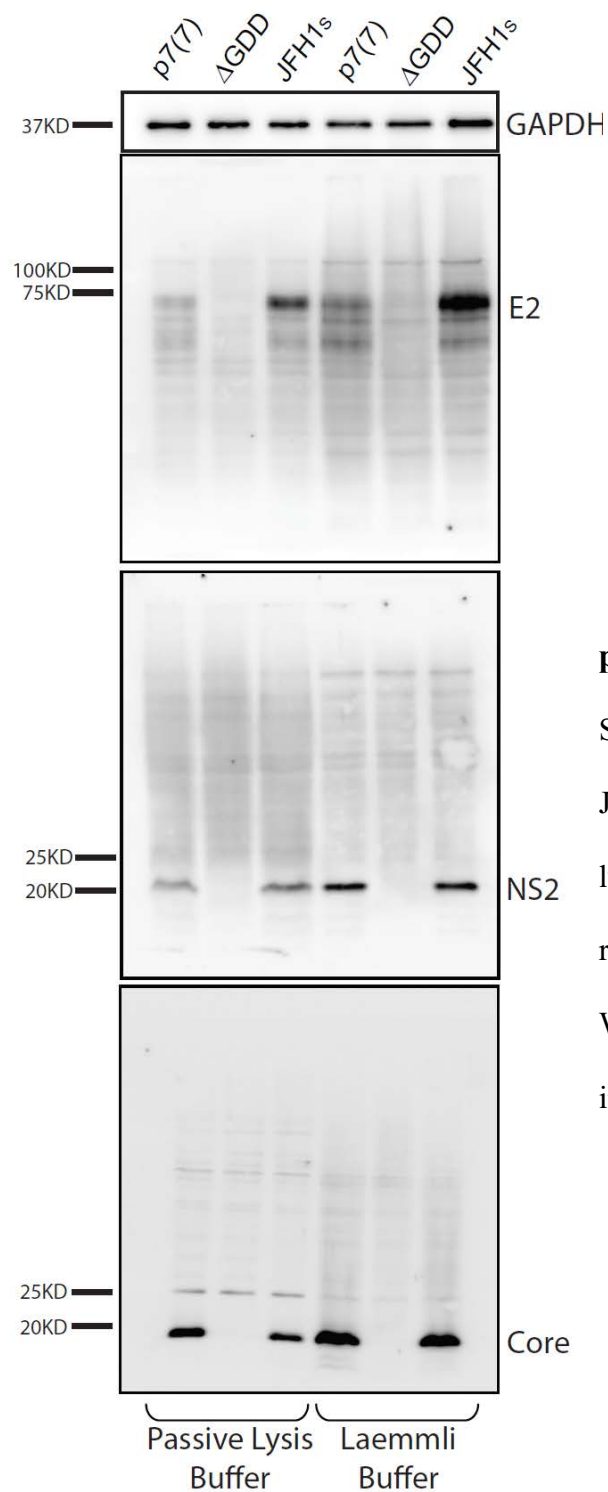
**Figure 4.8 Comparison of virus production from JFH1<sub>D</sub>, JFH1<sub>S</sub> and JFH1<sub>A4S</sub>.**

Huh-7.5 cells were transfected with the indicated constructs. 72 h later, culture supernatants were filtered and virus titers determined. Titrations were performed in triplicate and error bars represent standard error of the mean.

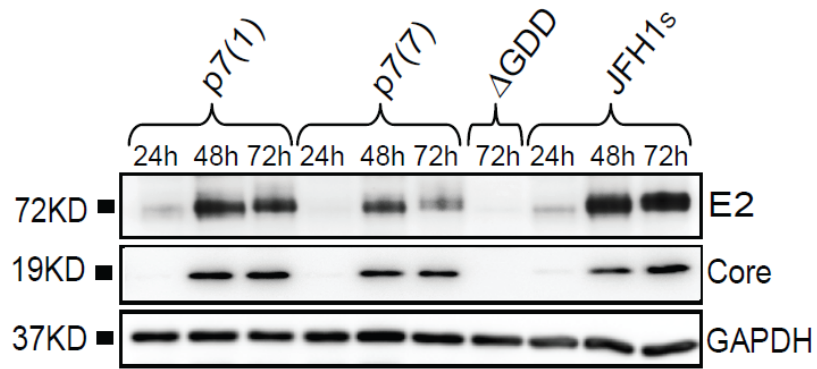


**Figure 4.9 Effects of p7 mutations on E2 levels.** (A) Huh-7.5 and S29 cells were transfected with the indicated p7 mutants generated in the background of JFH1<sub>A4S</sub> along with appropriate controls (JFH1<sub>A4S</sub> and ΔGDD). At 72 h post-transfection, intracellular lysates were obtained and probed with antibodies against E1, E2, NS2, core, and GAPDH by WB. (B) Band intensity measurements for core and E2 where each mutant's band intensity was calculated relative to JFH1<sub>A4S</sub>.

Previous reports have demonstrated that alteration of the p7 cytoplasmic loop can result in the detection of E2-p7-NS2 precursors by WB analysis [349]. While we did not observe this effect in Figure 4.7 or 4.9, E2 and NS2 blots were repeated using SDS-based Laemmli buffer in addition to our usual CHAPS-based buffer (Figure 4.10). Here, we also saw no evidence of precursors on either the E2 or NS2 protein blots. Therefore, we presume that the observed phenotype for p7(7) resulted from direct effects on the protein itself rather than a processing defect. To further investigate the observed reduction of E2 in the p7(7) mutant, we carried out a time course analysis of E2 levels at 24, 48, and 72 h post-transfection in S29 cells. Since S29 cells are non-permissive for HCV entry, they represent a relatively synchronized state of viral protein production upon transfection, which is ideally suited for this experiment. Here, the JFH1<sub>S</sub> strain of virus was used since it produces higher viral titers than JFH1<sub>A4S</sub> and detection of E1 protein was not required for this experiment. p7(1) was also included as an additional control since, like p7(7), it produces no infectious virus, yet generates near wild-type levels of E2. Lysates were prepared from each time-point and E2, core, and GAPDH levels were probed by WB (Figure 4.11). It was observed that at 24 h post-transfection the mutants and controls produced barely detectable levels of both E2 and core. By contrast, at the 48 h time-point, only p7(1) and the JFH1<sub>S<sub>A4S</sub></sub> control displayed comparable levels of core and E2, with a slightly reduced amount of E2 in the case of p7(7). However, E2 levels expressed from p7(7) were noticeably reduced by 72 h compared to what they were at 48 h, as well as being diminished compared to the other constructs tested.



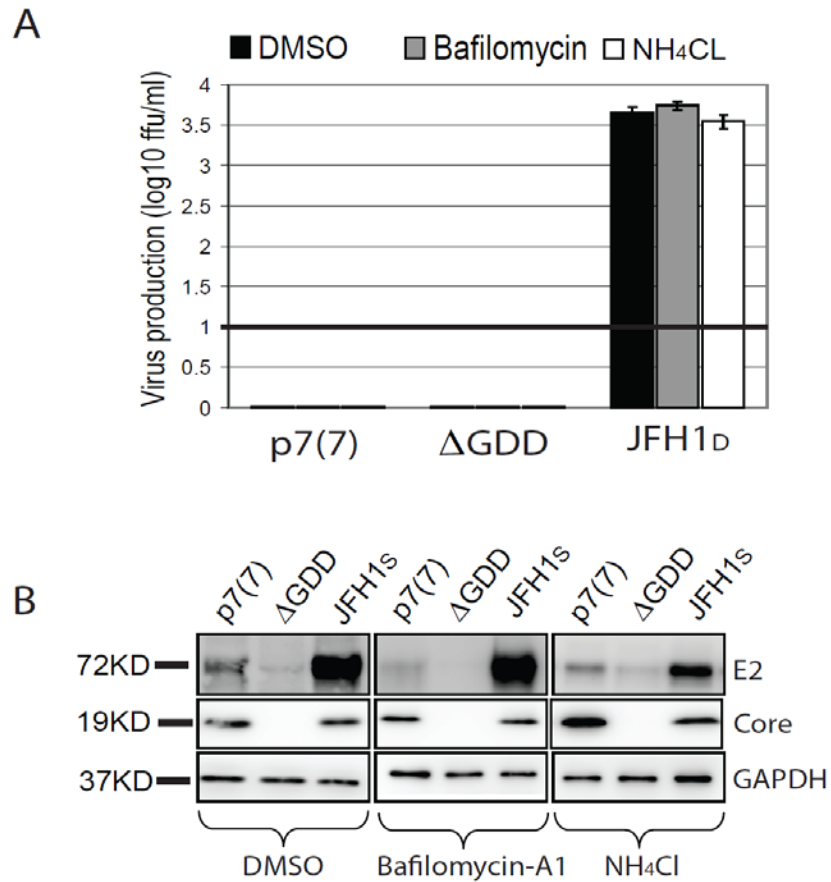
**Figure 4.10 Examination of HCV proteins under different lysis conditions.** S29 were transfected with RNA encoding JFH1<sub>s</sub>, ΔGDD or p7(7). 72 h later, cells were lysed and probed with antibodies recognizing core, NS2, E2 and GAPDH by WB. Results are representative of three independent experiments.



**Figure 4.11 Time course of E2, core, and GAPDH expression assessed by WB.**

Prepared lysates at 24, 48, and 72 h post-transfection of S29 cells for the mutants p7(1) and p7(7) generated in the background of JFH1<sub>s</sub> were collected and probed for the indicated viral proteins. Results are representative of two independent experiments.

In a previous report it was shown that expression of p7 or HCV infection prevents intracellular vesicle acidification, but this was not the case for the loop mutation [178]. This group also found that p7-induced loss of vesicle acidification contributes to infectious virus production as the p7 loop mutant genome transfection was partially rescued for infectious virus production after treating the cells with the acidification inhibitor bafilomycin A1. We interpreted this to mean that the observed reduction in E2 levels in the case of p7(7) was due to the loss of the ability of p7 to mediate the proper pH environment during the assembly process, resulting in E2 degradation. Therefore, we attempted to rescue both infectious virus production and E2 levels from p7(7) by treating transfected cells with (i) Bafilomycin A1, to inhibit vesicular acidification as performed previously [178], and (ii) ammonium chloride, a lysosomal inhibitor (Figure 4.12). However, we observed no restoration of virus production or E2 to levels seen with JFH1<sub>S</sub>. Taken together, these results indicated that the mutations present in p7(7) appear to result in lower levels of E2, which cannot be restored through the inhibition of lysosome or vesicular acidification (see discussion for further details).



**Figure 4.12 Effect of Bafilomycin A1 and NH<sub>4</sub>Cl treatment on E2 levels.** (A) Huh-7.5 cells were transfected with equivalent amounts of RNA representing p7(7) or control constructs (JFH1<sub>D</sub> and ΔGDD). At 48 h post-transfection, media were removed and replaced with media containing (i) 4 pM Bafilomycin A1, (ii) 12 mM NH<sub>4</sub>Cl or (iii) DMSO only. 24 h later, culture supernatants were filtered and virus titers determined. Note that serial dilution was performed in media containing the treatment agents. Titrations were performed in triplicate and error bars represent standard error of the mean. (B) S29 cells treated as above (here, JFH1<sub>S</sub> was used as a positive control instead of JFH1<sub>D</sub> in order to permit detection of E2) were lysed and probed for core, E2 and GAPDH by WB. Results are representative of three independent experiments.

## **CHAPTER 5: RESULTS (FORCED EVOLUTION ANALYSIS OF p7 MUTANTS)**

Results in this chapter have been submitted for publication while *Virology Journal*.

### **5.1 Passaging of p7 mutants led to adaptation and restoration of virus production by acquisition of amino acid changes on different viral proteins**

To further investigate the observed effect on infectious virus production of the p7 mutants generated, we passaged transfected cells with the generated p7 mutations 1-13 for multiple rounds in order to allow virus production-defective mutants to gain one or more compensatory mutations, which would provide genetic evidence for a possible p7 protein binding partner, or shed light on the amino acids playing the greatest role in p7 function. This strategy is illustrated in Figure 5.1 and has been employed by other groups [359,365]. Firstly, the viral spread on Huh-7.5 cells transfected with RNA of the respective mutants after each round lasting for 72 h was monitored. Upon confirmation of viral adaptation through indirect IF and observation of increased viral spread, passaging was terminated see Figure 5.1.

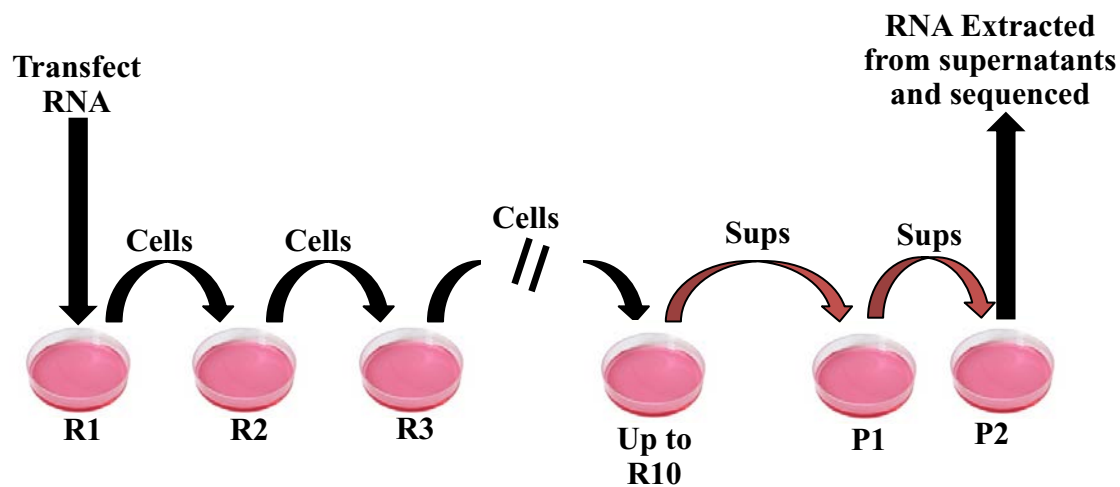
Mutation p7(1) showed a significant increase in viral spread at day 12 (R4) post-transfection. Efficient viral spread for the mutants p7(2) and p7(3) was observed at day 15 (R5) post-transfection. Mutant p7(6) completely regained efficient viral spread at day 30 (R10) post-transfection. Mutants p7(8) and p7(10) displayed efficient viral spread at day 18 (R6) and day 21 (R7) post-transfection, respectively. The mutations p7(4), p7(7), p7(9), p7(11), p7(12) and p7(13) were unable to restore virus production and passaging was terminated at the end of round 10 (Figure 5.2).



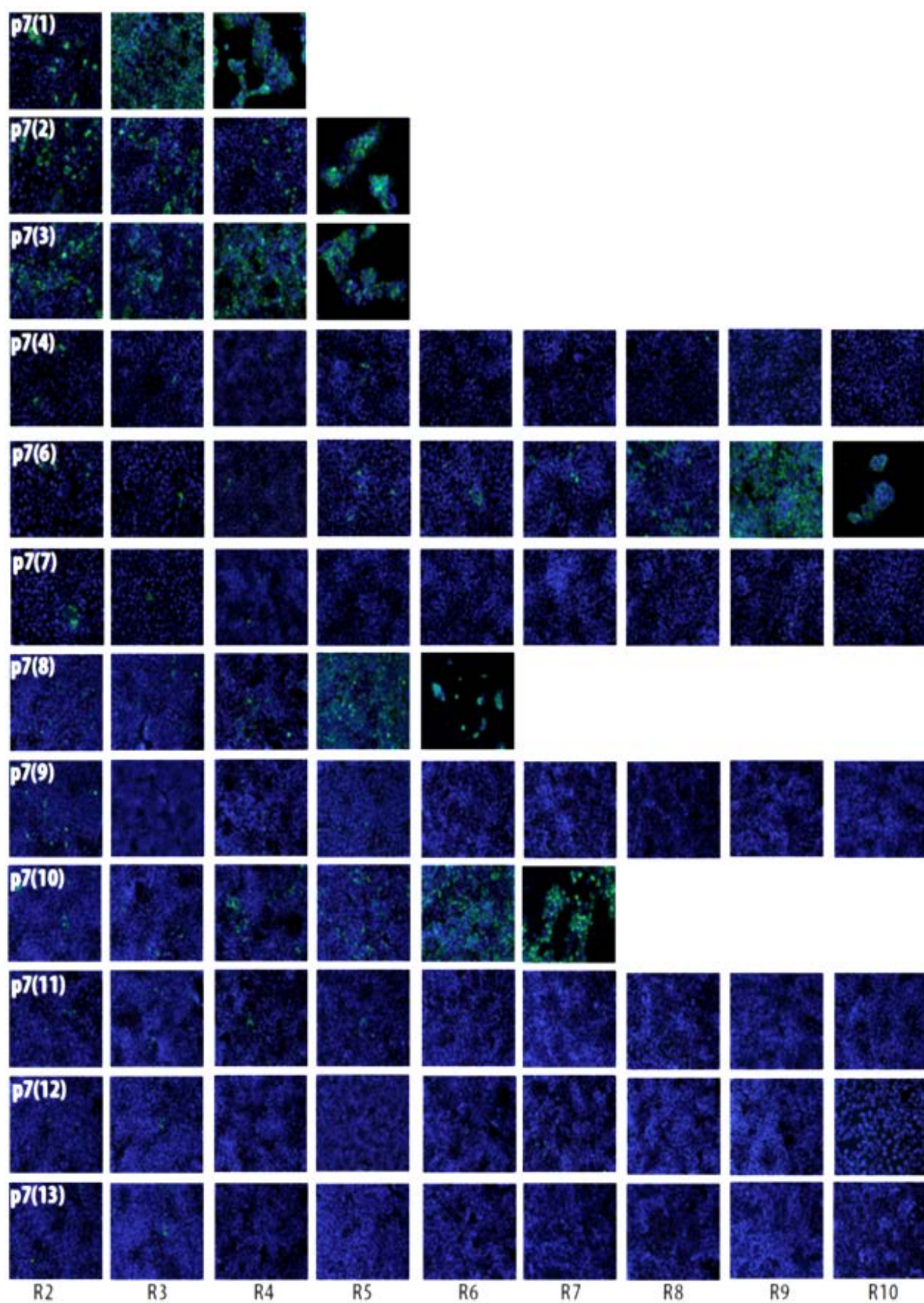
The mutants that showed a significant increase in virus spread were selected for determination of the consensus sequence of the polyprotein to identify the changes responsible for restoring viral kinetics. To do this, RNAs isolated from respective mutants were amplified by RT-PCR and sequenced, as described in Materials and Methods. The mutations we found are listed in Table 5.1.

## **5.2 The adaptive mutation N765D is important for p7 function**

One of the compensatory mutations we found was the N765D that significantly restored viral spread of p7(1) mutations. Aspartic acid was acquired at position 765 where the original amino acid in JFH1<sub>D</sub> or JFH-1 is asparagine; the alanine residues changed downstream remained unmodified throughout the passaging, indicating the importance of this amino acid for p7 function. The N765D mutation was reported before as an adaptive mutation that increases virus production by itself or combined with other mutations in E2 and NS2 [365]. In another report, N765D was found to restore virus production after a defect caused by exchange of the NS2 helix in the second TMD (trans membrane domain) of JFH-1 by that of the Con1 strain [65]. Therefore, we decided to further investigate the role of the N765D mutation in infectious virus production. Subsequent analyses of other mutations identified will be conducted by a future student.



**Figure 5.1 A schematic representation of the method used to identify compensatory mutations.** Huh-7.5 cells were transfected with RNAs encoding p7 mutant viruses. Cells were passaged each round for 72 h, and supernatants from the day of passage were saved to determine infectious titers. For R1 to R10, the cells themselves were passaged, and checked for viral spread by core IF as a 1<sup>st</sup> indicator of adaptation. Once improved virus spread was observed, supernatants were passaged for P1 and P2 to enrich for rescued virus. For P1, naive Huh-7.5 cells were infected using supernatants from the last round in which significant virus spread was observed. Seventy-two hours later, supernatants were removed and used to infect naive cells for a second passage (P2). RNA was harvested from P2 supernatants 72 h post-infection and used for sequencing. (Figure modified from [359])



**Figure 5.2 p7 mutant passaging.** IF core staining of p7 mutant passaging where each round lasted 72 h. Trypsinized cells after each round were resuspended in CM, seeded in 8-well chamber slides, and incubated for further 48 h. Slides were then stained for core (green) and nuclei (blue). Slides were examined using a fluorescent microscope at 20 X magnification.

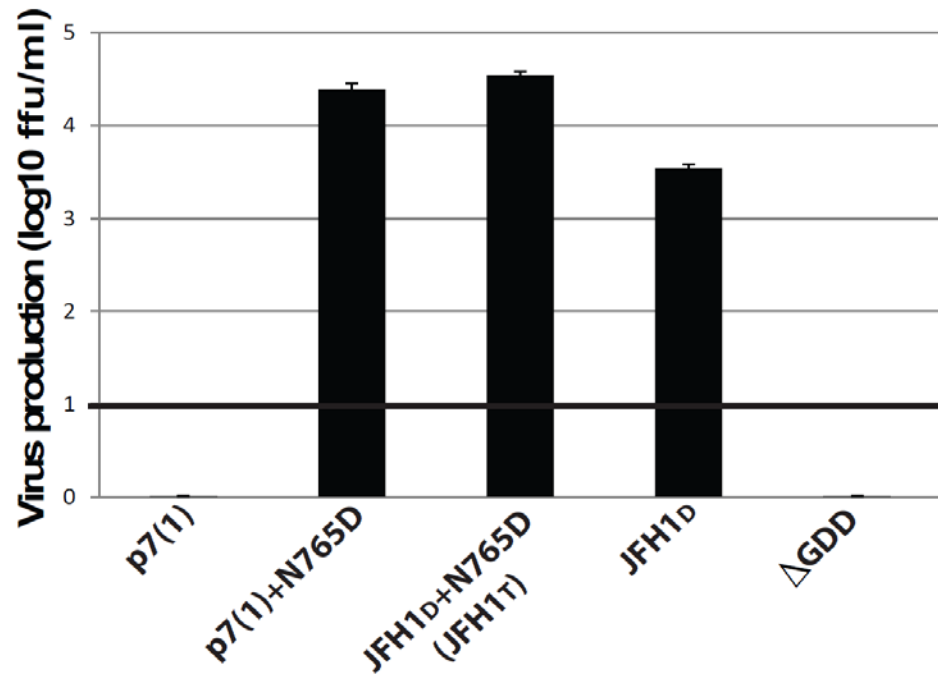
**Table 5.1 Sequence analysis of the rescued p7 mutants**

Mutation name	Rescue mutation (nt-position)	AA. Change	Location	Comments	Round and day number of daptation
p7(1)	C2633G	A765D	p7	Original aa is N	R4/day 12
	T2819C	L826L	NS2	Silent	
	T6450Y	V2022V/A	NS5A		
p7(2)	T2649Y	A770A/V	p7	Original aa is L	R5/day 15
	C3466T	G1042G	NS3	Silent	
	C3475T	G1045G	NS3	Silent	
	C4017M	T1226T/N	NS3		
	T6350C	F2004L	NS5A		
p7(3)	T2612G	L758V	p7		R5/day 15
	A3705G	K1122R	NS3		
	A7631R	T2431T/A	NS5A		
p7(6)	T2666C	F776L	p7		R10/day 30
	C7754A	L2472M	NS5B		
p7(8)	T910A	S190S	Core	Silent	R6/day 18
	G2637A	C766T	p7		
	C4593G	A1418G	NS3		
p7(10)	A924G	K194R	E1		R7/day 21
	A1130G	M264V	E1		
	T3035K	L899L/V	NS2		

RNAs were extracted from the virus stocks generated after long-term passage of the indicated p7 mutants (1<sup>st</sup> column), followed by cDNA synthesis, and full genome amplification and sequencing was performed. The resultant changes including nucleotides (nt), amino acids (aa) and locations are shown in the 2<sup>nd</sup>, 3<sup>rd</sup>, and 4<sup>th</sup> columns, respectively. The time at which each virus mutant showed a significant change in virus spread is indicated by round number and days post-transfection in the last column.

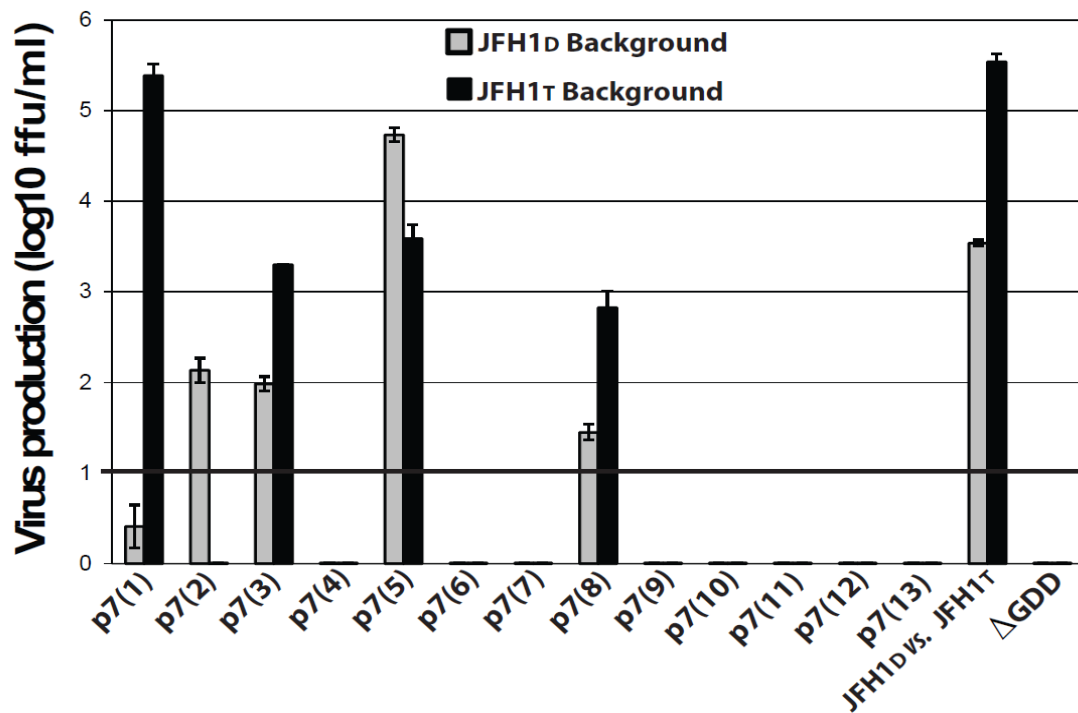
Firstly, we reintroduced N765D in the background of its cognate plasmid (p7(1)) or in the background of JFH1<sub>D</sub> to confirm if this adaptive mutation is responsible for the observed rescue of viral spread after passaging. We found that N765D significantly increased virus production and was the responsible mutation in the rescue of the triple alanine mutant at the p7(1) position (Figure 5.3). There were additional mutations found in the p7(1) rescued virus, but we did not further analyse these because it was clear that N765D was responsible for the restoration, and the silent nature of the remaining substitutions omitted them from analysis.

Next, we asked whether N765D was also sufficient for the other p7 mutations but could not be acquired through passaging because the virus might have been too weak to sustain itself in culture long enough to pick up rescue mutations. Accordingly, we recreated all p7 mutations (1 to 13) in the background of the maximal adapted strain of JFH-1 termed JFH1<sub>T</sub>, which has the two adaptive mutations previously described in JFH1<sub>D</sub> as well as the N765D. 72 h post-transfection of this panel of virus mutants, supernatants were collected and used to infect naïve Huh-7.5 cells in order to determine the effects on virus titer. We observed that N765D also restored virus production when introduced in the background of p7(3) and p7(8) genomes, but had no effect on the rest of the p7 mutations (Figure 5.4). This mutation was unfavorable for p7(2) and to a lesser extent for p7(5) as it reduced infectious virus production to undetectable levels compared with p7(2) and by one log compared with p7(5) in the background of JGH1<sub>D</sub>. These results indicated that N765D on p7 TM1 was able to restore alanine triplet changes at positions 765-767 (p7(1)), 771-773 (p7(3)) and 788-790 (p7(8)).



**Figure 5.3 Effect of the N765D adaptive mutation on infectious virus production.**

Huh-7.5 cells were transfected with the indicated constructs. 72 h later, culture supernatants were filtered and virus titers determined. Titrations were performed in triplicate and error bars represent standard error of the mean.



**Figure 5.4 Effect of the N765D adaptive mutation on infectious virus production for all of the generated p7 mutants.** Huh-7.5 cells were transfected with the indicated constructs. 72 h later, culture supernatants were filtered and virus titers determined. Titrations were performed in triplicate and error bars represent standard error of the mean.

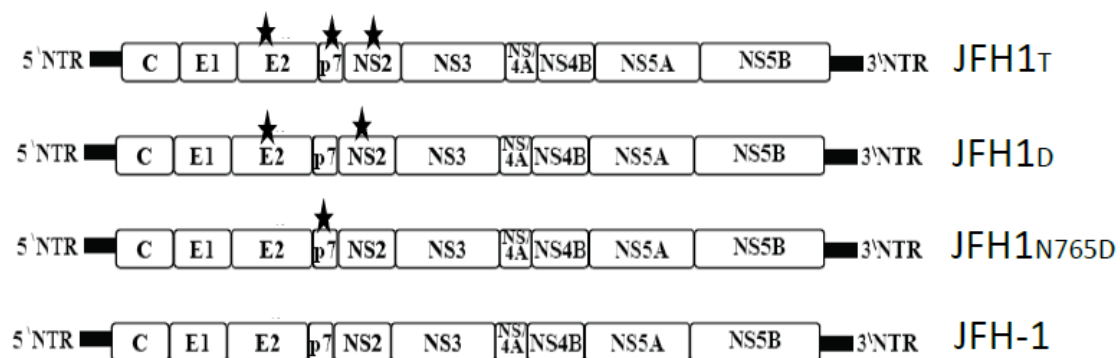


### **5.3 Additional analysis of N765D revealed a key role of p7 in efficient viral assembly and provided evidence against involvement in HCV entry processes.**

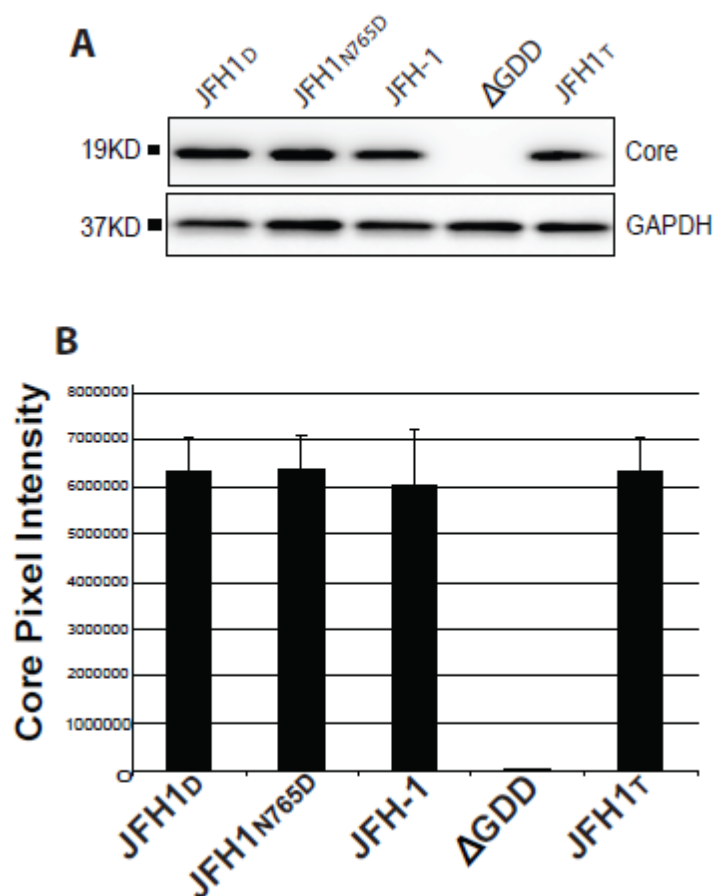
In a previous report N765D by itself increased infectious virus production 2 logs more than that of wild-type JFH-1, and when combined with the E2 and NS2 adaptive mutations, it increased virus production to 3 to 4 logs higher than wild-type [365]. Therefore, we took advantage of the ability of the N765D mutation to enhance virus production when combined with other adaptive mutations in order to further analyse the role of p7. Our hypothesis was that whatever p7 is doing, it can do it better in the presence of the N765D mutation. To test this hypothesis, we tested the effects of the presence and absence of N765D on the accumulation of virus particles in culture supernatants at 72 h post-transfection. In this case, we selected JFH1<sub>T</sub> (containing E2, p7 and NS2 adaptive mutations), JFH1<sub>D</sub> (containing only E2 and NS2 adaptive mutations), JFH1<sub>N765D</sub> (containing only the p7 N765D adaptive mutation) and wild type JFH-1 (Figure 5.5). We compared the levels of virus particles produced by these constructs in the culture supernatant after particle sedimentation through 20% sucrose cushion. Here, we also took advantage of the single-cycle virus production assay to allow for a comparison between mutants in a system that does not include virus entry steps. However, for results in this experiment to be valid, we needed to be sure that we had similar transfection efficiencies among constructs used. Therefore, we evaluated intracellular core protein expression by WB and flow cytometry analysis to ensure that all constructs used were comparable in transfection efficiencies (Figure 5.6).

Virus titration assays were then performed on all transfections and we confirmed that all constructs were competent for infectious virus production. As expected, we observed

1-2 log differences in virus production with JFH1<sub>T</sub> > JFH1<sub>D</sub> > JFH1<sub>N765D</sub> > wild type JFH-1. Culture supernatants were then also loaded at the top of a 20% sucrose cushion, which allows only assembled particles to pass through after short duration, but high velocity ultracentrifugation. WB analysis for core protein performed on pelleted virus demonstrated that extracellular HCV accumulation in JFH1<sub>T</sub> was more than that of JFH1<sub>D</sub> (Figure 5.7), and the only difference between these two constructs is the presence of the p7 adaptive mutation, N765D. Similarly, JFH1<sub>N765D</sub> accumulated more HCV particles than wild type JFH-1 with the difference between them also being the presence of the p7 N765D mutation. Taken together these results indicated that the N765D mutation in p7 enhanced the production of virus particles from transfected cells in an entry-null system. The interpretation of these findings, with some extrapolation, could be that if N765D enhances the function of p7, and that effect was observed in the increased production of virus in an entry-null system, then p7 likely is involved in the later stages of virus production, and not in the virus entry process.



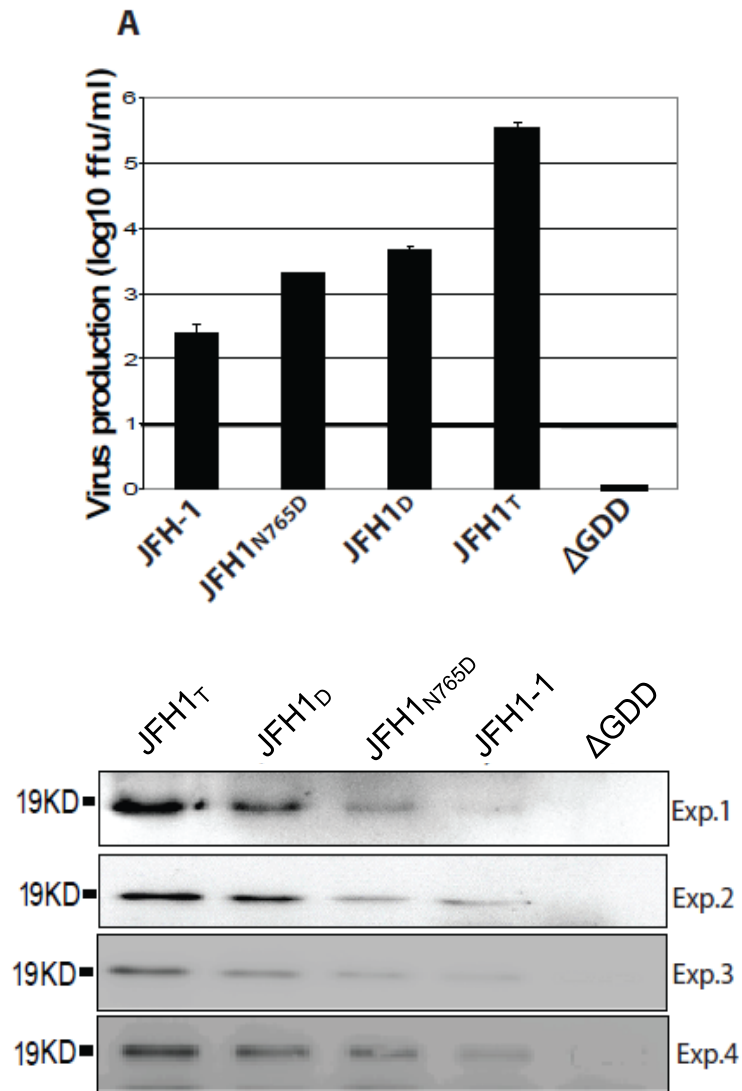
**Figure 5.5 Illustrative diagram for the constructs selected to analyze N765D adaptive mutation effect on extracellular HCV particles secretions.** Constructs names are shown at the right. The adaptive mutations E2 (N417S), p7 (N765D), and NS2 (Q1012R) are indicated by an asterisk on their respective genes.



**C**

Mutant	JFH1 <sub>T</sub>	JFH1 <sub>D</sub>	JFH1 <sub>N765D</sub>	JFH-1	ΔGDD
Trans. Eff.	9.1%	8.29%	7.95%	9.56%	0.36%

**Figure 5.6 Analysis of transfection efficiencies by WB and flow cytometry.** S29 cells were transfected with each construct indicated and 27 h later cells were pelleted, lysed and probed for intracellular core and GAPDH (A). Band intensity measurements for each sample from two independent transfection experiments were performed (B). Cell lysates were also stained for core protein and analyzed for percentages of core protein contents by flow cytometry (C).



**Figure 5.7 N765D increased the production of extracellular HCV particles. (A)** 72 h post-transfection S29 cell supernatants were clarified and used for infection titer measurements by ffu assay **(B)** or loaded over preformed 20% sucrose cushions, ultracentrifuged and the pellet probed for core protein by WB. Repeats of 4 independent experiments are shown.

## CHAPTER 6: DISCUSSION

The functionality of the p7 protein is one of the more poorly understood aspects of the HCV life cycle. p7 is important for both successful HCV infection in chimpanzees as well as virus production in cell culture, yet is seemingly dispensable for virus entry and RNA replication [300,301,306,321,348,349]. Multiple studies indicate that p7 forms ion channels in artificial membranes, leading to its inclusion in the viroporin protein family [308,316,318]. Notably, amino acids required for the ion channel activity of p7 were also shown to be important for pH modulation of intracellular vesicles in cell culture, and this activity was important for maintaining infectious virus production [178]. In this study, for the 1<sup>st</sup> time, we performed an extensive mutational analysis to determine at which stage of the viral life cycle p7 acts. Alanine triplet mutations spanning most of TM1, the cytoplasmic loop, and TM2 were generated and tested for their ability to produce virus, and subsequently, some of these mutations were tested at different stages of the viral life cycle. The data indicated that regions of TM1, the cytoplasmic loop and TM2 of p7 are important for virus production. p7 was not involved in virus release as reductions in both intra- and extracellular infectious virus were observed. Furthermore, mutating TM1 and the cytoplasmic loop of p7 did not alter core localization to LD, and capsid assembly was seemingly unaffected. Most importantly, we observed that alanine substitution of the two basic residues within the cytoplasmic loop of p7 caused a reduction in the amount of E2 present in transfected cells. Furthermore, we performed forced evolution analysis of p7 mutants by passaging of virus production incompetent mutants and provided more information on the most important amino acid composition of the p7 protein. We found

that the amino acid at position 765 is uniquely important for p7 function. Further analysis of the N765D substitution highlighted the importance of p7 in the HCV assembly process.

The 1<sup>st</sup> mutants analyzed in this study covered the TM1 domain of p7 were found to be critical for virus production, but to a lesser extent of the amino acids situated in the central region of TM1. This observation suggests that the positioning of the N- and C-termini of TM1 might be critical for anchoring the membrane-spanning region into the ER, and that mutation of this sequence may disrupt this positioning, thereby inhibiting virus production. Mutation of amino acids located near the N-terminal end of TM1 (p7(1)) caused complete abrogation of virus production. This result is in agreement with a study showing that the H17 residue (H767 in the polyprotein of JFH-1) within TM1 is important for virus production and is part of a HXXXW-like motif found in the influenza virus M2 protein, which is the main functional element of the M2 channels [321]. However, a separate study generated a single alanine substitution at residue N17 (N767 in the polyprotein of J6) in the context of the J6/JFH-1 chimeric virus and found an insignificant effect on virus production [300]. These discrepancies might be explained by the chimeric nature of the viral backbone used in the latter study, or may result from the triple alanine mutation studied here more effectively disrupting the M2-like motif. Interestingly, it has been previously reported that an adaptive mutation in this region (N15D; N765D in the JFH-1 polyprotein) enhanced virus production by 10-fold itself, further demonstrating the importance of these residues and suggesting that a putative interaction with other viral proteins is mediated by residues contained within the region mutated in p7(1) [365]. Mutations p7(2) and p7(3) lie within the central region of TM1 and reduced virus production to approximately 50% of the levels observed with JFH1<sub>D</sub>.

The alanines substituted in this region replace uncharged hydrophobic amino acids (GLLYFA) and we propose, therefore, that these substitutions likely alter the optimal structure of this segment, but not sufficiently enough to terminate virion production. Others have also found this region to have a minimal effect on virus production [300]. The p7(4) mutation completely abrogated virus production, indicating the importance of one or more amino acids in this region. Interestingly, others identified a compensatory mutation (F26L; also mutated in our p7(4) construct) that rescued a mutation in the core protein [364], indicating that p7 and core may work together through a direct interaction that has yet to be demonstrated. Thus, the targeted p7(4) residues might disrupt such an interaction. These results highlight that the integrity of both the N- and C-terminal regions of TM1 are important for the generation of infectious virus. It should also be noted that, as is usually the case for alanine-scanning mutagenic analyses, some of the constructed mutations were more conservative than others. For example, it was unsurprising that p7(5) (mutation from VAA to AAV) exhibited no reduction in virus production, most likely since the overall folding of p7 was largely unaltered due to the conservative changes being introduced. This would not be the case for some of the other mutants discussed later where more drastic mutations were made, including p7(6) (mutation from WHI to AAA) and p7(7) (mutation from RGR to AAA). In the case of these latter mutants, we cannot exclude that virus production is abrogated due to p7 structural deformities resulting from the non-conservative amino acid changes.

The data herein also showed that the two basic residues of the cytoplasmic loop of p7, R33 and R35, were important for infectious virus production. The importance of the cytoplasmic loop in virus production was shown previously in multiple studies employing



the HCVcc system, and *in vivo* after intrahepatic injection of chimpanzees [300,348,349]. Most of these studies concentrated on the two basic residues (R33 and R35), since it was previously shown that these residues were important for p7 ion channel activity in artificial membrane assays [308,316]. In this study we performed a comprehensive, side-by-side comparison of viruses containing mutations covering most of the loop sequence. We observed that the p7(7) mutant, which included the dibasic residues discussed above, and mutation p7(6), which is situated at the junction of the cytoplasmic loop, both terminated virus production. Moreover, a previous study created a W30F mutation, within the region that was also mutated in our p7(6), and found to be an important residue for p7 function and virus production [349]. Therefore, we have confirmed the importance of the cytoplasmic loop structure in virus production.

Similarly, we also showed that TM2 of p7 is important for virus production in cell culture. In agreement with a previous report showing that a mutation in p7 TM2 (Y42F) (included in p7(9) in our study) reduced infectious virus production and release [349]. On the other hand, St. Gelais *et al.* showed that amino acid residues that might affect ion channel activity or drug resistance in p7, including the mutations G39A (J4 sequence) (included in p7(8)) and P49A (included in p7(9)), cause a specific ion channel formation defect. This group also indicated that Pro 49 may serve to regulate efficient oligomerization of p7 channel complexes. Furthermore, in the same report, a L(50-55)A mutation (included in p7(12) and p7(13)) did not affect ion channel oligomerization but showed differential sensitivity to the ion channel blockers amantadine and rimantadine, highlighting the importance of this region of p7 in ion channel structure and drug sensitivity [320]. So, our study was the first comprehensive study showing the importance

of TM2 in infectious virus production. It was noticeable that TM2 mutations showed more drastic effects on virus production compared to TM1 mutations. One possible explanation for these differences are that TM2 may be more important for p7 structure and function. This also could be explained by the nature of amino acid changes that we introduced, *i.e.*, the alanine changes had less impact on TM1 membrane integration but disrupted TM2 insertion through the ER membrane.

In order to identify at which stage of the viral life cycle p7 play its role, we tested only the mutations at TM1 and the cytoplasmic loop at multiple steps of the virus life cycle. During the early stages of virus assembly, core protein traffics to the surface of LDs, which are proposed to serve as a platform for virion formation [96]. Thus, we have tested our mutation at this stage and conclusively demonstrated that none of the p7 mutants tested affected core protein accumulation around LD. One recent study proposed that the core/LD association results from inefficient virus assembly and that efficiently assembling virus strain Jc1 did not accumulate significant levels of core/LD association. [355]. However, this was not the case in our study as the efficiently-assembling JFH1<sub>D</sub>, as well as all virus production-defective mutants, showed equivalent core/LD association. Another report employed the less efficient LD core accumulating strain Jc1. They found that mutations in the p7 cytoplasmic loop affected the unloading of core protein from LDs, resulting in a retention of core around LDs after quantitation of core on isolated cellular LDs by flotation through a discontinuous density gradient [30]. It was also found through partial and full gene swaps that compatibilities between p7 and the first NS2 trans-membrane domains were required to induce core-ER localization [355]. Correspondingly, other studies visualized that p7 can manipulate the intracellular

distribution of NS2 and disrupt its binding pattern with other viral proteins [172]. Moreover, a study tracking core protein in live cells found that the movements of cytoplasmic labelled-core puncta were dependent on NS2 function [170]. Ultimately, a p7-NS2 interaction with the NS3-4A complex was shown to be important for viral protein colocalization patterns and was suggested to perform the unloading process of core protein from LD into sites of virus assembly [174]. To sum up, p7 could be an important secondary factor for unloading of core protein from LD toward further assembly processes where NS2 protein is the major player, but it requires assistance through a p7 interaction.

The molecular details of HCV assembly are currently under intense investigation and are a matter of much debate. Following the recruitment of core to LDs, HCV capsid presumably begins to assemble through oligomerization of core, forming virus particles associated with a copy of the viral genome. This step presumably involves the production of multiple structures including oligomerized core representing both newly-forming nucleocapsids, and end-stage nucleocapsids associated with triglycerides and  $\beta$ -lipoprotein (VLDL and LDL). Evidence for this model is supported by the observed pattern of core distribution within density gradient analyses [41,358,359,375]. In order to determine whether p7 plays a direct role in nucleocapsid assembly, we performed iodixanol gradient analyses on a select panel of p7 mutant viruses from cells that were lysed with a detergent-containing buffer. Based on the data shown, we can conclude with reasonable confidence that p7 does not affect core assembly because we were able to detect dense species of core protein in fractions 6-8 of the gradient. Our findings are in agreement with another group who performed similar gradient fractionation experiments

[376]. However, one study performed quantitation of core protein in the prepared fractions and found an increase in the proportion of incompletely assembled capsids indicating defective and delayed formation of intracellular core structures [30]. In fact, in this report it is not clear whether the accumulation of unassembled core containing fractions were due to impaired p7 effects on nucleocapsid assembly or if it is an effect due to impaired virus production in general, as p7 mutant genomes and controls showed comparable core concentrations in the fractions that were suggested to contain assembled core species. Also, the density measurements observed in the gradient fraction that contained the infectious particles were significantly higher (1.35 g/ml) than what is reported in the literature, with HCVcc particle densities ranging between 1.0 to 1.18 g/ml [41-45]. However, different protocols were employed, i.e., sucrose-based rate zonal centrifugation, whereas we used iodixanol equilibrium gradients. It would be interesting to determine whether we can identify nucleocapsid assembly defects in our mutants by rate zonal centrifugation. In the end, the available data to date indicate that p7 is likely not involved in formation of multi-order structures of core during nucleocapsid assembly.

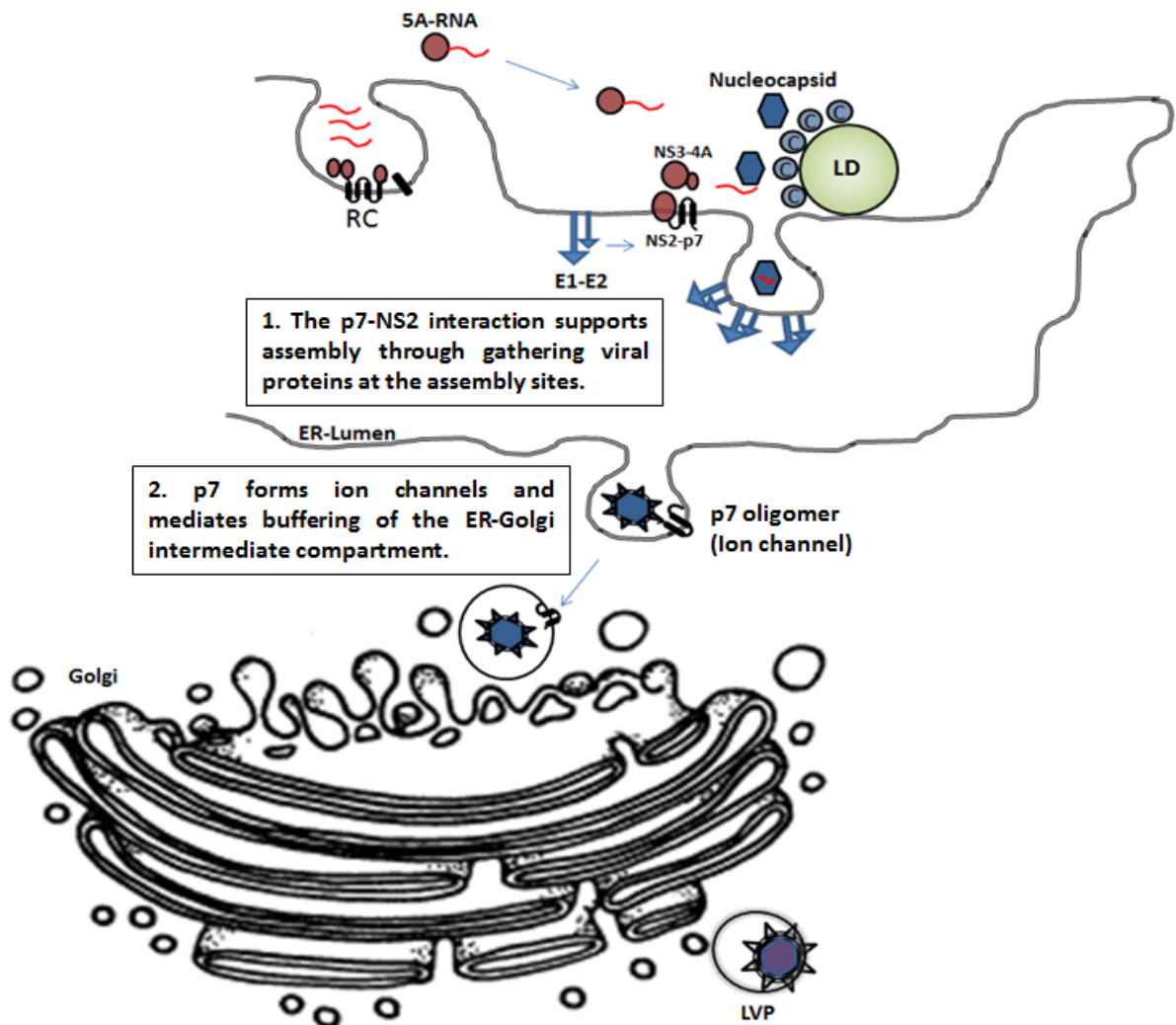
Finally, Wozniak *et al.* found that p7 modulates intracellular proton conductance and increases lysosomal pH from 4.5 to 6.0 during HCV infection in cell culture [178]. This report also demonstrated that the acidification inhibitor Bafilomycin A1 and expression of influenza virus M2 protein restored virus production from a HCV genome mutated at the basic residues of the p7 cytoplasmic loop. Presumably, such a function would be important in protecting newly-formed virions from premature degradation at a late stage in virion production, specifically the envelope glycoproteins. We now show that a mutation within the loop that includes the same two basic residues also affected E2

protein expression and/or stability. Bafilomycin A1 and ammonium chloride did not restore virus production in our case. The Bafilomycin A1 rescue demonstrated in the previous report was partial and the mutation generated was a double alanine change of the two p7 basic residues. In addition, the replication foci were noticeably smaller in cells inoculated with the rescued mutant virus and the treatments were unable to rescue the delta p7 genome [178]. Therefore, in our case we suggested that the triple alanine changes at p7(7) possibly caused a complete abrogation of p7 ion channel activity to an extent that could not be rescued by the above mentioned treatments. However, it is still unclear why this effect on E2 was only observed in the context of the cytoplasmic loop mutation (p7 (7)). One theory is that p7 plays a dual role in the late viral assembly process; on one hand p7 could mediate proper targeting of viral glycoproteins to the assembly site in collaboration with NS2, and we suggested that in case of the mutations p7(1-6) envelope protein is still retained in a protective environment that did not move to a site of further assembly due to the disruption of p7-NS2 interaction. On the other hand, p7 then could protect immature glycoproteins from degradation where envelope protein starts to be exposed to an unfavorable environment through an ion channel-like activity during late assembly or release. This theory is supported by a study showing that pseudoreversion in p7 within TM1 of p7 (N15D) combined with a mutation at NS2 (G25R) restored virus infectivity and colocalization of NS2 around LDs along with E2 and NS3 [65]. These effects were not a consequence of p7 ion channel function [356]. Also, Ma *et al.* showed that p7 deletion and mutations within the cytoplasmic loop affected the intracellular distribution of NS2 and E2 [172]. Similarly, Staplford *et al.* found that NS2 interaction with other viral proteins was dependent on p7 coexpression [66]. Furthermore, Vieyres *et*

*al.* recently constructed a functional HCV genome with a HA-tagged p7 and found that p7 interacted with NS2 and colocalized with E2 on the ER membrane [306]. Therefore, it seems that p7 function may play two roles in HCV life cycle where it is required for NS2 function in coordinating viral protein trafficking during the assembly process, while in the second role, it protects virion glycoprotein E2 from immature degradation through an ion channel-like activity disrupted by the p7(7) mutation studied herein. It is worth mentioning here that the HCV envelope proteins acquire high mannose and complex N-linked glycans through post-translational modification, which usually occurs in the Golgi apparatus [176]. Also, envelope proteins rearrange the disulphide bonds necessary to prime HCV particles for low-pH mediated fusion, similar to pestivirus egress through the Golgi [131,176,177]. The trafficking of HCV particles and pre-envelope maturation is thought to happen within intracellular vesicles. At this stage, the viral protein p7 ion channel activity might be required to protect viral envelope proteins during passaging through Golgi.

After passaging of p7 mutants by forced evolution assays, we found that one of the previously-identified adaptive mutations (N765D) amazingly also emerged as a compensatory mutation for some of our loss-of-function mutants and was able to restore alanine triplet changes on positions 765-767 (p7(1)), 771-773 (p7(3)) and 788-790 (p7(8)). This indicates the importance of this residue in p7 structure and function. The N765D mutation was reported before as an adaptive mutation that increases virus production by itself or in combination with other adaptive mutations in E2 and NS2 [365]. In another report, N765D was found to restore virus production after a defect caused by exchange of a NS2 helix in the second TMD (trans membrane domain) of JFH-

1 by that of the Con1 strain [65]. Therefore, it seems this mutation is not only critical for p7 structure but also important for interaction engagements with other viral proteins such as NS2. We believe that the N-terminal end of p7 interacts with the second transmembrane domain of NS2. The further analysis we performed on the adaptive mutation N765D indicated its importance for efficient accumulation of HCV particles in the culture supernatants. This indicates that p7 might act at the assembly stage and/or release but less likely at the entry process as these data were obtained in the entry-null S29 cell single-cycle assay. This finding is supported by multiple reports showing that HCV pseudo-particle entry occurs independent of p7 [350,351] and, later by indirect evidence, showing that the specific infectivity of p7 mutants was not reduced compared to wild-type virus [349]. Also, another study created an infectious construct containing a double HA-tagged p7 and found that p7 was not contained within the virion, which it would presumably be if it were required for entry, even in concentrated, affinity-purified or Flag-tagged preparations of virus [306]. Unexpectedly, introducing N765D in the background of HCV genome containing the mutation p(2) was not favorable and led to complete reduction of infectious virus, which could indicate that the functionality of certain amino acids may be influenced by its adjacent residues. However, it would be of significant interest to determine whether p7 structural features and ion channel activity are preserved in the presence of the alanine substitutions that were rescued by the N765D mutation, which would provide indirect evidence for the potential ion channel function.



**Figure 6.1 Proposed model of p7 functions.** Illustrative diagram of p7 function integrated into the HCV assembly process. The newly synthesized genome is delivered by NS4A to the site of nucleocapsid formation and NS3-4A supports genome packaging. Concurrently, the E1-E2 complex with ER-derived membrane makes up the viral envelope. The p7-NS2 complex orchestrates the recruitment of viral proteins to the site of assembly. Also, p7 forms an ion channel that mediate proper pH of virion-containing vesicles in order to protect immature virions until complete envelope glycoprotein maturation has taken place in the Golgi.



**Conclusion:**

p7 is required for *in vivo* infectivity, and important for infectious virus production in cell culture. For unknown reasons, the cleavage of p7 from adjacent viral proteins is delayed, leading to the presence of precursor polypeptides (E2-p7, p7-NS2 or E2-p7-NS2) proposed to have regulatory roles in virus production. Recent structural and topological studies have confirmed that p7 mainly localizes to the ER and contains two trans-membrane domains connected by a short cytoplasmic loop. By oligomerizing in the membrane, p7 is believed to form an ion channel that facilitates proton permeabilization in order to equilibrate intracellular vesicle pH to promote infectious virus production. The ion-channel activity can be inhibited by several compounds, with BIT225 and iminosugar derivatives showing the most promising results. Moreover, p7 interacts with NS2 and supports a late step in viral assembly and envelopment. The interaction pattern of p7 with other viral and host factors, and its exact contribution to infectious virus production remains poorly defined.

In our study we confirmed, in a comprehensive mutational study that p7 is an important factor in virus production, specifically at a late stage in viral assembly, and it might be that p7 is a dual function protein. In one aspect, p7, presumably in its monomeric form, assists NS2 in gathering newly-formed capsids at LDs and glycoprotein complexes on the ER lumen for proper envelopment. Whereas, in its oligomeric state, p7 protects glycoproteins from immature degradation during trafficking and release through an ion channel-like activity. A proposed model of p7 function in the HCV assembly process is shown in the Figure 6.1.

**Future directions:**

To continue this work, we are interested to further analyze the generated p7 mutations' effects on various stages of the HCV life cycle, particularly at the assembly and release steps. Initially, we would like to test the p7 mutants' sedimentation profiles by rate zonal centrifugation using sucrose gradients to be able to confirm whether p7 is involved in the formation of multi-order structures, such as HCV nucleocapsids. By doing this, we will have a clear image on the best way to perform gradient fractionations on HCV core protein.

We are also interested in creating single alanine mutation exchanges of the two basic residues of the cytoplasmic loop and determine whether bafilomycin A1, NH<sub>4</sub>Cl, or p7 expression *in trans* can rescue virus production. We also plan to perform time course analyses for E2 levels with pulse-chase assays. Doing such experiments will give us an idea if neutralization of intracellular vesicles contributes to infectious virus production and support our notion that p7 mediates E2 protection from premature degradation during HCV morphogenesis.

It's very important to study the interaction pattern of p7 with other viral proteins. However, the small size of p7 and its membrane topology makes it a poorly immunogenic protein and, therefore, it is difficult to generate a reliable antibody against it. The idea of adding tag sequences to such a small protein might interfere with the actual binding pattern or the membrane topology of p7. It is possible that peptide antibody against p7 could be generated using the carrier protein Keyhole limpet hemocyanin (KLH). Using this approach, synthetic peptides of only the accessible parts of p7 (the N- and C-termini and the cytoplasmic loop) would be the best choices to be conjugated with KLH. In

addition, studying the effect of p7 mutations on different viral protein localization and distribution by IF, particularly NS2 and E2 proteins, would be the best choice to gain more insight into the effect of p7 on other viral proteins, and would shed more light on the specific role of p7.

Other adaptive mutations were also found to rescue viral spread in Huh-7.5 cells (mutations p7(2), p7(3), p7(6), p7(8), p7(10)) (see Table 5.1). Analysis of the identified mutations in the manner we used for the N765D will also provide important information on the most important residues of the p7 sequence and how certain viral proteins might have contributed to the rescue of the p7 defects. The N765D mutation was able to rescue alanine triplet changes at positions 765-767 (p7(1)), 771-773 (p7(3)) and 788-790 (p7(8)). We think it would be important to test whether these triple alanine mutants harboring N765D also preserve ion channel activity in artificial membrane assays. In this way, we could confirm the existence of the putative ion channel activity of p7.

Looking a little deeper, it was reported that ion channel proteins of the non-enveloped viruses (togaviruses and lentiviruses) and the enveloped viruses (coronaviruses) mediate changes of host membrane potentials and promote viral budding at the plasma membrane or at an intracellular vesicle membrane in a process called depolarization-dependent exocytosis [310]. Coronaviruses are enveloped viruses with positive sense RNA genomes that also encode a viroporin, termed E protein. This viroporin was shown to mediate membrane rearrangements and formation of electron dense membrane structures derived from ER-Golgi intermediate compartments (ERGIC) and was suggested to play a role in scission of particles at the ERGIC [313]. It has never been considered whether p7 might mediate E protein-like activity. It would be exciting to test whether expression of p7

mediates the same membrane effects and modification of membrane potential. It would also be interesting to determine whether trafficking and release of HCV particles relies on ESCRT or COPII pathways or if p7 protein mediates this process.

We firmly believe that p7 represents an attractive target for novel HCV treatment due to its small size and its importance for HCV infection. A better understanding of its function is of the utmost importance for development of a comprehensive therapeutic regimen.

## REFERENCES

1. Colombo M (1999) Natural history and pathogenesis of hepatitis C virus related hepatocellular carcinoma. *J Hepatol* 31 Suppl 1: 25-30.
2. Mohd Hanafiah K, Groeger J, Flaxman AD, Wiersma ST (2013) Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. *Hepatology* 57: 1333-1342.
3. Davis GL, Esteban-Mur R, Rustgi V, Hoefs J, Gordon SC, et al. (1998) Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. *N Engl J Med* 339: 1493-1499.
4. Patel K, McHutchison JG (2004) Initial treatment for chronic hepatitis C: current therapies and their optimal dosing and duration. *Cleve Clin J Med* 71 Suppl 3: S8-12.
5. Manns MP, Wedemeyer H, Cornberg M (2006) Treating viral hepatitis C: efficacy, side effects, and complications. *Gut* 55: 1350-1359.
6. Jacobson IM, McHutchison JG, Dusheiko G, Di Bisceglie AM, Reddy KR, et al. (2011) Telaprevir for previously untreated chronic hepatitis C virus infection. *N Engl J Med* 364: 2405-2416.
7. Poordad F, McCone J, Jr., Bacon BR, Bruno S, Manns MP, et al. (2011) Boceprevir for untreated chronic HCV genotype 1 infection. *N Engl J Med* 364: 1195-1206.
8. Sarrazin C, Hezode C, Zeuzem S, Pawlotsky JM (2012) Antiviral strategies in hepatitis C virus infection. *J Hepatol* 56 Suppl 1: S88-100.
9. Feinstone SM, Kapikian AZ, Purcell RH, Alter HJ, Holland PV (2001) Transfusion-associated hepatitis not due to viral hepatitis type A or B. 1975. *Rev Med Virol* 11: 3-8; discussion 8-9.
10. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, et al. (1989) Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 244: 359-362.
11. Kuo G, Choo QL, Alter HJ, Gitnick GL, Redeker AG, et al. (1989) An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. *Science* 244: 362-364.
12. Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, et al. (1990) Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc Natl Acad Sci U S A* 87: 9524-9528.
13. Tanaka T, Kato N, Cho MJ, Shimotohno K (1995) A novel sequence found at the 3' terminus of hepatitis C virus genome. *Biochem Biophys Res Commun* 215: 744-749.
14. Choo QL, Richman KH, Han JH, Berger K, Lee C, et al. (1991) Genetic organization and diversity of the hepatitis C virus. *Proc Natl Acad Sci U S A* 88: 2451-2455.
15. Kaito M, Watanabe S, Tsukiyama-Kohara K, Yamaguchi K, Kobayashi Y, et al. (1994) Hepatitis C virus particle detected by immunoelectron microscopic study. *J Gen Virol* 75 ( Pt 7): 1755-1760.
16. Li X, Jeffers LJ, Shao L, Reddy KR, de Medina M, et al. (1995) Identification of hepatitis C virus by immunoelectron microscopy. *J Viral Hepat* 2: 227-234.
17. Shimizu YK, Feinstone SM, Kohara M, Purcell RH, Yoshikura H (1996) Hepatitis C virus: detection of intracellular virus particles by electron microscopy. *Hepatology* 23: 205-209.
18. Kato N, Hijikata M, Nakagawa M, Ootsuyama Y, Muraiso K, et al. (1991) Molecular structure of the Japanese hepatitis C viral genome. *FEBS Lett* 280: 325-328.
19. Reed KE, Rice CM (2000) Overview of hepatitis C virus genome structure, polyprotein processing, and protein properties. *Curr Top Microbiol Immunol* 242: 55-84.

20. Kapoor A, Simmonds P, Gerold G, Qaisar N, Jain K, et al. (2011) Characterization of a canine homolog of hepatitis C virus. *Proc Natl Acad Sci U S A* 108: 11608-11613.
21. Kapoor A, Simmonds P, Scheel TK, Hjelle B, Cullen JM, et al. (2013) Identification of rodent homologs of hepatitis C virus and pegiviruses. *MBio* 4: e00216-00213.
22. Quan PL, Firth C, Conte JM, Williams SH, Zambrana-Torrel CM, et al. (2013) Bats are a major natural reservoir for hepaciviruses and pegiviruses. *Proc Natl Acad Sci U S A* 110: 8194-8199.
23. Gottwein JM, Scheel TK, Jensen TB, Lademann JB, Prentoe JC, et al. (2009) Development and characterization of hepatitis C virus genotype 1-7 cell culture systems: role of CD81 and scavenger receptor class B type I and effect of antiviral drugs. *Hepatology* 49: 364-377.
24. Kuiken C, Simmonds P (2009) Nomenclature and numbering of the hepatitis C virus. *Methods Mol Biol* 510: 33-53.
25. Domingo E, Martinez-Salas E, Sobrino F, de la Torre JC, Portela A, et al. (1985) The quasispecies (extremely heterogeneous) nature of viral RNA genome populations: biological relevance--a review. *Gene* 40: 1-8.
26. Kato N (2001) Molecular virology of hepatitis C virus. *Acta Med Okayama* 55: 133-159.
27. Hijikata M, Kato N, Ootsuyama Y, Nakagawa M, Shimotohno K (1991) Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. *Proc Natl Acad Sci U S A* 88: 5547-5551.
28. Hijikata M, Mizushima H, Akagi T, Mori S, Kakiuchi N, et al. (1993) Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. *J Virol* 67: 4665-4675.
29. Hijikata M, Mizushima H, Tanji Y, Komoda Y, Hirowatari Y, et al. (1993) Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. *Proc Natl Acad Sci U S A* 90: 10773-10777.
30. Gentzsch J, Brohm C, Steinmann E, Friesland M, Menzel N, et al. (2013) hepatitis c Virus p7 is critical for capsid assembly and envelopment. *PLoS Pathog* 9: e1003355.
31. Atoom AM, Jones DM, Russell RS (2013) Evidence suggesting that HCV p7 protects E2 glycoprotein from premature degradation during virus production. *Virus Res* 176: 199-210.
32. Lindenbach BD, Rice CM (2013) The ins and outs of hepatitis C virus entry and assembly. *Nat Rev Microbiol* 11: 688-700.
33. Dediu S, Voiculescu M, Zamfirescu I, Perederi L, State D (1974) [Prolonged viral hepatitis: patterns of evolution and prognosis (clinico-biochemical and morphological comparisons in 25 cases)]. *Med Interna (Bucur)* 26: 611-616.
34. Lukavsky PJ, Otto GA, Lancaster AM, Sarnow P, Puglisi JD (2000) Structures of two RNA domains essential for hepatitis C virus internal ribosome entry site function. *Nat Struct Biol* 7: 1105-1110.
35. Spangberg K, Wiklund L, Schwartz S (2001) Binding of the La autoantigen to the hepatitis C virus 3' untranslated region protects the RNA from rapid degradation in vitro. *J Gen Virol* 82: 113-120.
36. Mukhopadhyay S, Kuhn RJ, Rossmann MG (2005) A structural perspective of the flavivirus life cycle. *Nat Rev Microbiol* 3: 13-22.
37. Nielsen SU, Bassendine MF, Burt AD, Martin C, Pumeechokchai W, et al. (2006) Association between hepatitis C virus and very-low-density lipoprotein (VLDL)/LDL analyzed in iodixanol density gradients. *J Virol* 80: 2418-2428.

38. Thomssen R, Bonk S, Propfe C, Heermann KH, Kochel HG, et al. (1992) Association of hepatitis C virus in human sera with beta-lipoprotein. *Med Microbiol Immunol* 181: 293-300.
39. Kono Y, Hayashida K, Tanaka H, Ishibashi H, Harada M (2003) High-density lipoprotein binding rate differs greatly between genotypes 1b and 2a/2b of hepatitis C virus. *J Med Virol* 70: 42-48.
40. Diaz O, Delers F, Maynard M, Demignot S, Zoulim F, et al. (2006) Preferential association of Hepatitis C virus with apolipoprotein B48-containing lipoproteins. *J Gen Virol* 87: 2983-2991.
41. Gastaminza P, Kapadia SB, Chisari FV (2006) Differential biophysical properties of infectious intracellular and secreted hepatitis C virus particles. *J Virol* 80: 11074-11081.
42. Merz A, Long G, Hiet MS, Brugger B, Chlanda P, et al. (2011) Biochemical and morphological properties of hepatitis C virus particles and determination of their lipidome. *J Biol Chem* 286: 3018-3032.
43. Catanese MT, Uryu K, Kopp M, Edwards TJ, Andrus L, et al. (2013) Ultrastructural analysis of hepatitis C virus particles. *Proc Natl Acad Sci U S A* 110: 9505-9510.
44. Chang KS, Jiang J, Cai Z, Luo G (2007) Human apolipoprotein e is required for infectivity and production of hepatitis C virus in cell culture. *J Virol* 81: 13783-13793.
45. Meunier JC, Russell RS, Engle RE, Faulk KN, Purcell RH, et al. (2008) Apolipoprotein c1 association with hepatitis C virus. *J Virol* 82: 9647-9656.
46. Meex SJ, Andreo U, Sparks JD, Fisher EA (2011) Huh-7 or HepG2 cells: which is the better model for studying human apolipoprotein-B100 assembly and secretion? *J Lipid Res* 52: 152-158.
47. Bartenschlager R, Penin F, Lohmann V, Andre P (2011) Assembly of infectious hepatitis C virus particles. *Trends Microbiol* 19: 95-103.
48. Boulant S, Montserret R, Hope RG, Ratniner M, Targett-Adams P, et al. (2006) Structural determinants that target the hepatitis C virus core protein to lipid droplets. *J Biol Chem* 281: 22236-22247.
49. Boulant S, Vanbelle C, Ebel C, Penin F, Lavergne JP (2005) Hepatitis C virus core protein is a dimeric alpha-helical protein exhibiting membrane protein features. *J Virol* 79: 11353-11365.
50. Roingeard P, Hourieux C (2008) Hepatitis C virus core protein, lipid droplets and steatosis. *J Viral Hepat* 15: 157-164.
51. Tsutsumi T, Matsuda M, Aizaki H, Moriya K, Miyoshi H, et al. (2009) Proteomics analysis of mitochondrial proteins reveals overexpression of a mitochondrial protein chaperon, prohibitin, in cells expressing hepatitis C virus core protein. *Hepatology* 50: 378-386.
52. Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, et al. (1998) The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med* 4: 1065-1067.
53. Grakoui A, Wychowski C, Lin C, Feinstone SM, Rice CM (1993) Expression and identification of hepatitis C virus polyprotein cleavage products. *J Virol* 67: 1385-1395.
54. Lanford RE, Notvall L, Chavez D, White R, Frenzel G, et al. (1993) Analysis of hepatitis C virus capsid, E1, and E2/NS1 proteins expressed in insect cells. *Virology* 197: 225-235.
55. Owsianka AM, Timms JM, Tarr AW, Brown RJ, Hickling TP, et al. (2006) Identification of conserved residues in the E2 envelope glycoprotein of the hepatitis C virus that are critical for CD81 binding. *J Virol* 80: 8695-8704.
56. Voisset C, Callens N, Blanchard E, Op De Beeck A, Dubuisson J, et al. (2005) High density lipoproteins facilitate hepatitis C virus entry through the scavenger receptor class B type I. *J Biol Chem* 280: 7793-7799.

57. Kato N, Ootsuyama Y, Tanaka T, Nakagawa M, Nakazawa T, et al. (1992) Marked sequence diversity in the putative envelope proteins of hepatitis C viruses. *Virus Res* 22: 107-123.
58. Steinmann E, Pietschmann T (2010) Hepatitis C virus p7-a viroporin crucial for virus assembly and an emerging target for antiviral therapy. *Viruses* 2: 2078-2095.
59. Lorenz IC, Marcotrigiano J, Dentzer TG, Rice CM (2006) Structure of the catalytic domain of the hepatitis C virus NS2-3 protease. *Nature* 442: 831-835.
60. Pietschmann T, Kaul A, Koutsoudakis G, Shavinskaya A, Kallis S, et al. (2006) Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. *Proc Natl Acad Sci U S A* 103: 7408-7413.
61. Kolykhalov AA, Mihalik K, Feinstone SM, Rice CM (2000) Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3' nontranslated region are essential for virus replication in vivo. *J Virol* 74: 2046-2051.
62. Dimitrova M, Imbert I, Kieny MP, Schuster C (2003) Protein-protein interactions between hepatitis C virus nonstructural proteins. *J Virol* 77: 5401-5414.
63. Yi M, Ma Y, Yates J, Lemon SM (2009) Trans-complementation of an NS2 defect in a late step in hepatitis C virus (HCV) particle assembly and maturation. *PLoS Pathog* 5: e1000403.
64. Jirasko V, Montserret R, Appel N, Janvier A, Eustachi L, et al. (2008) Structural and functional characterization of nonstructural protein 2 for its role in hepatitis C virus assembly. *J Biol Chem* 283: 28546-28562.
65. Jirasko V, Montserret R, Lee JY, Gouttenoire J, Moradpour D, et al. (2010) Structural and functional studies of nonstructural protein 2 of the hepatitis C virus reveal its key role as organizer of virion assembly. *PLoS Pathog* 6: e1001233.
66. Stapleford KA, Lindenbach BD (2011) Hepatitis C virus NS2 coordinates virus particle assembly through physical interactions with the E1-E2 glycoprotein and NS3-NS4A enzyme complexes. *J Virol* 85: 1706-1717.
67. Love RA, Parge HE, Wickersham JA, Hostomsky Z, Habuka N, et al. (1996) The crystal structure of hepatitis C virus NS3 proteinase reveals a trypsin-like fold and a structural zinc binding site. *Cell* 87: 331-342.
68. Tai CL, Chi WK, Chen DS, Hwang LH (1996) The helicase activity associated with hepatitis C virus nonstructural protein 3 (NS3). *J Virol* 70: 8477-8484.
69. Kim JL, Morgenstern KA, Lin C, Fox T, Dwyer MD, et al. (1996) Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. *Cell* 87: 343-355.
70. Wolk B, Sansonno D, Krausslich HG, Dammacco F, Rice CM, et al. (2000) Subcellular localization, stability, and trans-cleavage competence of the hepatitis C virus NS3-NS4A complex expressed in tetracycline-regulated cell lines. *J Virol* 74: 2293-2304.
71. Lindenbach BD, Rice CM (2005) Unravelling hepatitis C virus replication from genome to function. *Nature* 436: 933-938.
72. Foy E, Li K, Wang C, Sumpter R, Jr., Ikeda M, et al. (2003) Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. *Science* 300: 1145-1148.
73. Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, et al. (2005) Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 437: 1167-1172.
74. Sen GC, Sarkar SN (2005) Hitching RIG to action. *Nat Immunol* 6: 1074-1076.
75. Li K, Foy E, Ferreon JC, Nakamura M, Ferreon AC, et al. (2005) Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc Natl Acad Sci U S A* 102: 2992-2997.



76. Kim JL, Morgenstern KA, Griffith JP, Dwyer MD, Thomson JA, et al. (1998) Hepatitis C virus NS3 RNA helicase domain with a bound oligonucleotide: the crystal structure provides insights into the mode of unwinding. *Structure* 6: 89-100.
77. Yao N, Hesson T, Cable M, Hong Z, Kwong AD, et al. (1997) Structure of the hepatitis C virus RNA helicase domain. *Nat Struct Biol* 4: 463-467.
78. Dubuisson J (2007) Hepatitis C virus proteins. *World J Gastroenterol* 13: 2406-2415.
79. Ma Y, Yates J, Liang Y, Lemon SM, Yi M (2008) NS3 helicase domains involved in infectious intracellular hepatitis C virus particle assembly. *J Virol* 82: 7624-7639.
80. Lundin M, Monne M, Widell A, Von Heijne G, Persson MA (2003) Topology of the membrane-associated hepatitis C virus protein NS4B. *J Virol* 77: 5428-5438.
81. Egger D, Wolk B, Gosert R, Bianchi L, Blum HE, et al. (2002) Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J Virol* 76: 5974-5984.
82. Einav S, Sklan EH, Moon HM, Gehrig E, Liu P, et al. (2008) The nucleotide binding motif of hepatitis C virus NS4B can mediate cellular transformation and tumor formation without Ha-ras co-transfection. *Hepatology* 47: 827-835.
83. Einav S, Elazar M, Danieli T, Glenn JS (2004) A nucleotide binding motif in hepatitis C virus (HCV) NS4B mediates HCV RNA replication. *J Virol* 78: 11288-11295.
84. Gouttenoire J, Penin F, Moradpour D (2010) Hepatitis C virus nonstructural protein 4B: a journey into unexplored territory. *Rev Med Virol* 20: 117-129.
85. Brass V, Bieck E, Montserret R, Wolk B, Hellings JA, et al. (2002) An amino-terminal amphipathic alpha-helix mediates membrane association of the hepatitis C virus nonstructural protein 5A. *J Biol Chem* 277: 8130-8139.
86. Hinson ER, Cresswell P (2009) The antiviral protein, viperin, localizes to lipid droplets via its N-terminal amphipathic alpha-helix. *Proc Natl Acad Sci U S A* 106: 20452-20457.
87. Tellinghuisen TL, Marcotrigiano J, Rice CM (2005) Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. *Nature* 435: 374-379.
88. Gale M, Jr., Blakely CM, Kwieciszewski B, Tan SL, Dossett M, et al. (1998) Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: molecular mechanisms of kinase regulation. *Mol Cell Biol* 18: 5208-5218.
89. Kanda T, Steele R, Ray R, Ray RB (2009) Inhibition of intrahepatic gamma interferon production by hepatitis C virus nonstructural protein 5A in transgenic mice. *J Virol* 83: 8463-8469.
90. Foster TL, Galloway P, Stonehouse NJ, Harris M (2011) Cyclophilin A interacts with domain II of hepatitis C virus NS5A and stimulates RNA binding in an isomerase-dependent manner. *J Virol* 85: 7460-7464.
91. Coelmont L, Hanouille X, Chatterji U, Berger C, Snoeck J, et al. (2010) DEB025 (Alisporivir) inhibits hepatitis C virus replication by preventing a cyclophilin A induced cis-trans isomerisation in domain II of NS5A. *PLoS One* 5: e13687.
92. Hanouille X, Badillo A, Wieruszewski JM, Verdegem D, Landrieu I, et al. (2009) Hepatitis C virus NS5A protein is a substrate for the peptidyl-prolyl cis/trans isomerase activity of cyclophilins A and B. *J Biol Chem* 284: 13589-13601.
93. Appel N, Zayas M, Miller S, Krijnse-Locker J, Schaller T, et al. (2008) Essential role of domain III of nonstructural protein 5A for hepatitis C virus infectious particle assembly. *PLoS Pathog* 4: e1000035.
94. Tellinghuisen TL, Foss KL, Treadaway J (2008) Regulation of hepatitis C virion production via phosphorylation of the NS5A protein. *PLoS Pathog* 4: e1000032.

95. Kaneko T, Tanji Y, Satoh S, Hijikata M, Asabe S, et al. (1994) Production of two phosphoproteins from the NS5A region of the hepatitis C viral genome. *Biochem Biophys Res Commun* 205: 320-326.
96. Miyanari Y, Atsuzawa K, Usuda N, Watashi K, Hishiki T, et al. (2007) The lipid droplet is an important organelle for hepatitis C virus production. *Nat Cell Biol* 9: 1089-1097.
97. Appel N, Pietschmann T, Bartenschlager R (2005) Mutational analysis of hepatitis C virus nonstructural protein 5A: potential role of differential phosphorylation in RNA replication and identification of a genetically flexible domain. *J Virol* 79: 3187-3194.
98. Evans MJ, Rice CM, Goff SP (2004) Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication. *Proc Natl Acad Sci U S A* 101: 13038-13043.
99. Kriegs M, Burckstummer T, Himmelsbach K, Bruns M, Frelin L, et al. (2009) The hepatitis C virus non-structural NS5A protein impairs both the innate and adaptive hepatic immune response in vivo. *J Biol Chem* 284: 28343-28351.
100. Lee C (2013) Daclatasvir: potential role in hepatitis C. *Drug Des Devel Ther* 7: 1223-1233.
101. Schmidt-Mende J, Bieck E, Hugle T, Penin F, Rice CM, et al. (2001) Determinants for membrane association of the hepatitis C virus RNA-dependent RNA polymerase. *J Biol Chem* 276: 44052-44063.
102. Lesburg CA, Cable MB, Ferrari E, Hong Z, Mannarino AF, et al. (1999) Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. *Nat Struct Biol* 6: 937-943.
103. Bressanelli S, Tomei L, Roussel A, Incitti I, Vitale RL, et al. (1999) Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. *Proc Natl Acad Sci U S A* 96: 13034-13039.
104. Koch U, Narjes F (2006) Allosteric inhibition of the hepatitis C virus NS5B RNA dependent RNA polymerase. *Infect Disord Drug Targets* 6: 31-41.
105. Jopling CL (2008) Regulation of hepatitis C virus by microRNA-122. *Biochem Soc Trans* 36: 1220-1223.
106. Henke JI, Goergen D, Zheng J, Song Y, Schuttler CG, et al. (2008) microRNA-122 stimulates translation of hepatitis C virus RNA. *EMBO J* 27: 3300-3310.
107. Song Y, Friebe P, Tzima E, Junemann C, Bartenschlager R, et al. (2006) The hepatitis C virus RNA 3'-untranslated region strongly enhances translation directed by the internal ribosome entry site. *J Virol* 80: 11579-11588.
108. Ye L, Timani KA, Ye L, Kong L, Yang X, et al. (2005) Two cis-acting elements in negative RNA strand of Hepatitis C virus involved in synthesis of positive RNA strand in vitro. *Acta Virol* 49: 83-90.
109. Otto GA, Puglisi JD (2004) The pathway of HCV IRES-mediated translation initiation. *Cell* 119: 369-380.
110. Saito T, Owen DM, Jiang F, Marcotrigiano J, Gale M, Jr. (2008) Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA. *Nature* 454: 523-527.
111. Barth H, Schafer C, Adah MI, Zhang F, Linhardt RJ, et al. (2003) Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. *J Biol Chem* 278: 41003-41012.
112. Agnello V, Abel G, Elfahal M, Knight GB, Zhang QX (1999) Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. *Proc Natl Acad Sci U S A* 96: 12766-12771.

113. Owen DM, Huang H, Ye J, Gale M, Jr. (2009) Apolipoprotein E on hepatitis C virion facilitates infection through interaction with low-density lipoprotein receptor. *Virology* 394: 99-108.
114. Scarselli E, Ansuini H, Cerino R, Roccasecca RM, Acali S, et al. (2002) The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J* 21: 5017-5025.
115. Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, et al. (1998) Binding of hepatitis C virus to CD81. *Science* 282: 938-941.
116. Sharma NR, Mateu G, Dreux M, Grakoui A, Cosset FL, et al. (2011) Hepatitis C virus is primed by CD81 protein for low pH-dependent fusion. *J Biol Chem* 286: 30361-30376.
117. Cormier EG, Tsamis F, Kajumo F, Durso RJ, Gardner JP, et al. (2004) CD81 is an entry coreceptor for hepatitis C virus. *Proc Natl Acad Sci U S A* 101: 7270-7274.
118. Dao Thi VL, Granier C, Zeisel MB, Guerin M, Mancip J, et al. (2012) Characterization of hepatitis C virus particle subpopulations reveals multiple usage of the scavenger receptor BI for entry steps. *J Biol Chem* 287: 31242-31257.
119. Zahid MN, Turek M, Xiao F, Thi VL, Guerin M, et al. (2013) The postbinding activity of scavenger receptor class B type I mediates initiation of hepatitis C virus infection and viral dissemination. *Hepatology* 57: 492-504.
120. Evans MJ, von Hahn T, Tscherne DM, Syder AJ, Panis M, et al. (2007) Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* 446: 801-805.
121. Ploss A, Evans MJ, Gaysinskaya VA, Panis M, You H, et al. (2009) Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature* 457: 882-886.
122. Harris HJ, Davis C, Mullins JG, Hu K, Goodall M, et al. (2010) Claudin association with CD81 defines hepatitis C virus entry. *J Biol Chem* 285: 21092-21102.
123. Dorner M, Horwitz JA, Robbins JB, Barry WT, Feng Q, et al. (2011) A genetically humanized mouse model for hepatitis C virus infection. *Nature* 474: 208-211.
124. Dorner M, Horwitz JA, Donovan BM, Labitt RN, Budell WC, et al. (2013) Completion of the entire hepatitis C virus life cycle in genetically humanized mice. *Nature* 501: 237-241.
125. Lupberger J, Zeisel MB, Xiao F, Thumann C, Fofana I, et al. (2011) EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. *Nat Med* 17: 589-595.
126. Blanchard E, Belouzard S, Goueslain L, Wakita T, Dubuisson J, et al. (2006) Hepatitis C virus entry depends on clathrin-mediated endocytosis. *J Virol* 80: 6964-6972.
127. Sabahi A (2009) Hepatitis C Virus entry: the early steps in the viral replication cycle. *Virol J* 6: 117.
128. Rey FA, Heinz FX, Mandl C, Kunz C, Harrison SC (1995) The envelope glycoprotein from tick-borne encephalitis virus at 2 Å resolution. *Nature* 375: 291-298.
129. El Omari K, Iourin O, Harlos K, Grimes JM, Stuart DI (2013) Structure of a pestivirus envelope glycoprotein E2 clarifies its role in cell entry. *Cell Rep* 3: 30-35.
130. Li Y, Wang J, Kanai R, Modis Y (2013) Crystal structure of glycoprotein E2 from bovine viral diarrhoea virus. *Proc Natl Acad Sci U S A* 110: 6805-6810.
131. Tscherne DM, Jones CT, Evans MJ, Lindenbach BD, McKeating JA, et al. (2006) Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry. *J Virol* 80: 1734-1741.
132. Koutsoudakis G, Kaul A, Steinmann E, Kallis S, Lohmann V, et al. (2006) Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. *J Virol* 80: 5308-5320.

133. Flint M, McKeating JA (1999) The C-terminal region of the hepatitis C virus E1 glycoprotein confers localization within the endoplasmic reticulum. *J Gen Virol* 80 ( Pt 8): 1943-1947.
134. Garry RF, Dash S (2003) Proteomics computational analyses suggest that hepatitis C virus E1 and pestivirus E2 envelope glycoproteins are truncated class II fusion proteins. *Virology* 307: 255-265.
135. Yagnik AT, Lahm A, Meola A, Roccasecca RM, Ercole BB, et al. (2000) A model for the hepatitis C virus envelope glycoprotein E2. *Proteins* 40: 355-366.
136. Mizushima H, Hijikata M, Tanji Y, Kimura K, Shimotohno K (1994) Analysis of N-terminal processing of hepatitis C virus nonstructural protein 2. *J Virol* 68: 2731-2734.
137. Lin C, Lindenbach BD, Pragai BM, McCourt DW, Rice CM (1994) Processing in the hepatitis C virus E2-NS2 region: identification of p7 and two distinct E2-specific products with different C termini. *J Virol* 68: 5063-5073.
138. Lo SY, Masiarz F, Hwang SB, Lai MM, Ou JH (1995) Differential subcellular localization of hepatitis C virus core gene products. *Virology* 213: 455-461.
139. Mizushima H, Hijikata M, Asabe S, Hirota M, Kimura K, et al. (1994) Two hepatitis C virus glycoprotein E2 products with different C termini. *J Virol* 68: 6215-6222.
140. Grakoui A, McCourt DW, Wychowski C, Feinstone SM, Rice CM (1993) A second hepatitis C virus-encoded proteinase. *Proc Natl Acad Sci U S A* 90: 10583-10587.
141. Grakoui A, McCourt DW, Wychowski C, Feinstone SM, Rice CM (1993) Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. *J Virol* 67: 2832-2843.
142. Tomei L, Failla C, Santolini E, De Francesco R, La Monica N (1993) NS3 is a serine protease required for processing of hepatitis C virus polyprotein. *J Virol* 67: 4017-4026.
143. D'Souza ED, O'Sullivan E, Amphlett EM, Rowlands DJ, Sangar DV, et al. (1994) Analysis of NS3-mediated processing of the hepatitis C virus non-structural region in vitro. *J Gen Virol* 75 ( Pt 12): 3469-3476.
144. Bartenschlager R, Ahlborn-Laake L, Yasargil K, Mous J, Jacobsen H (1995) Substrate determinants for cleavage in cis and in trans by the hepatitis C virus NS3 proteinase. *J Virol* 69: 198-205.
145. Komoda Y, Hijikata M, Tanji Y, Hirowatari Y, Mizushima H, et al. (1994) Processing of hepatitis C viral polyprotein in *Escherichia coli*. *Gene* 145: 221-226.
146. Komoda Y, Hijikata M, Sato S, Asabe S, Kimura K, et al. (1994) Substrate requirements of hepatitis C virus serine proteinase for intermolecular polypeptide cleavage in *Escherichia coli*. *J Virol* 68: 7351-7357.
147. Mackenzie J (2005) Wrapping things up about virus RNA replication. *Traffic* 6: 967-977.
148. Gosert R, Egger D, Lohmann V, Bartenschlager R, Blum HE, et al. (2003) Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. *J Virol* 77: 5487-5492.
149. Raney KD, Sharma SD, Moustafa IM, Cameron CE (2010) Hepatitis C virus non-structural protein 3 (HCV NS3): a multifunctional antiviral target. *J Biol Chem* 285: 22725-22731.
150. Tellinghuisen TL, Foss KL, Treadaway JC, Rice CM (2008) Identification of residues required for RNA replication in domains II and III of the hepatitis C virus NS5A protein. *J Virol* 82: 1073-1083.
151. Wang QM, Hockman MA, Staschke K, Johnson RB, Case KA, et al. (2002) Oligomerization and cooperative RNA synthesis activity of hepatitis C virus RNA-dependent RNA polymerase. *J Virol* 76: 3865-3872.

152. Weng L, Hirata Y, Arai M, Kohara M, Wakita T, et al. (2010) Sphingomyelin activates hepatitis C virus RNA polymerase in a genotype-specific manner. *J Virol* 84: 11761-11770.
153. Yang F, Robotham JM, Nelson HB, Irsigler A, Kenworthy R, et al. (2008) Cyclophilin A is an essential cofactor for hepatitis C virus infection and the principal mediator of cyclosporine resistance in vitro. *J Virol* 82: 5269-5278.
154. Watashi K, Ishii N, Hijikata M, Inoue D, Murata T, et al. (2005) Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Mol Cell* 19: 111-122.
155. Kim SJ, Kim JH, Kim YG, Lim HS, Oh JW (2004) Protein kinase C-related kinase 2 regulates hepatitis C virus RNA polymerase function by phosphorylation. *J Biol Chem* 279: 50031-50041.
156. Hamamoto I, Nishimura Y, Okamoto T, Aizaki H, Liu M, et al. (2005) Human VAP-B is involved in hepatitis C virus replication through interaction with NS5A and NS5B. *J Virol* 79: 13473-13482.
157. Barba G, Harper F, Harada T, Kohara M, Goulinet S, et al. (1997) Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. *Proc Natl Acad Sci U S A* 94: 1200-1205.
158. Ohsaki Y, Cheng J, Suzuki M, Shinohara Y, Fujita A, et al. (2009) Biogenesis of cytoplasmic lipid droplets: from the lipid ester globule in the membrane to the visible structure. *Biochim Biophys Acta* 1791: 399-407.
159. Boulant S, Douglas MW, Moody L, Budkowska A, Targett-Adams P, et al. (2008) Hepatitis C virus core protein induces lipid droplet redistribution in a microtubule- and dynein-dependent manner. *Traffic* 9: 1268-1282.
160. Menzel N, Fischl W, Hueging K, Bankwitz D, Frentzen A, et al. (2012) MAP-kinase regulated cytosolic phospholipase A2 activity is essential for production of infectious hepatitis C virus particles. *PLoS Pathog* 8: e1002829.
161. Herker E, Harris C, Hernandez C, Carpentier A, Kaehlcke K, et al. (2010) Efficient hepatitis C virus particle formation requires diacylglycerol acyltransferase-1. *Nat Med* 16: 1295-1298.
162. Penin F, Brass V, Appel N, Ramboarina S, Montserret R, et al. (2004) Structure and function of the membrane anchor domain of hepatitis C virus nonstructural protein 5A. *J Biol Chem* 279: 40835-40843.
163. Pietschmann T, Zayas M, Meuleman P, Long G, Appel N, et al. (2009) Production of infectious genotype 1b virus particles in cell culture and impairment by replication enhancing mutations. *PLoS Pathog* 5: e1000475.
164. Matto M, Rice CM, Aroeti B, Glenn JS (2004) Hepatitis C virus core protein associates with detergent-resistant membranes distinct from classical plasma membrane rafts. *J Virol* 78: 12047-12053.
165. Aizaki H, Morikawa K, Fukasawa M, Hara H, Inoue Y, et al. (2008) Critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection. *J Virol* 82: 5715-5724.
166. Andre P, Perlemuter G, Budkowska A, Brechot C, Lotteau V (2005) Hepatitis C virus particles and lipoprotein metabolism. *Semin Liver Dis* 25: 93-104.
167. Shelness GS, Sellers JA (2001) Very-low-density lipoprotein assembly and secretion. *Curr Opin Lipidol* 12: 151-157.
168. Nielsen SU, Bassendine MF, Burt AD, Bevitt DJ, Toms GL (2004) Characterization of the genome and structural proteins of hepatitis C virus resolved from infected human liver. *J Gen Virol* 85: 1497-1507.

169. Gastaminza P, Cheng G, Wieland S, Zhong J, Liao W, et al. (2008) Cellular determinants of hepatitis C virus assembly, maturation, degradation, and secretion. *J Virol* 82: 2120-2129.
170. Collier KE, Heaton NS, Berger KL, Cooper JD, Saunders JL, et al. (2012) Molecular determinants and dynamics of hepatitis C virus secretion. *PLoS Pathog* 8: e1002466.
171. Dubuisson J, Hsu HH, Cheung RC, Greenberg HB, Russell DG, et al. (1994) Formation and intracellular localization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia and Sindbis viruses. *J Virol* 68: 6147-6160.
172. Ma Y, Anantpadma M, Timpe JM, Shanmugam S, Singh SM, et al. (2011) Hepatitis C virus NS2 protein serves as a scaffold for virus assembly by interacting with both structural and nonstructural proteins. *J Virol* 85: 86-97.
173. Phan T, Beran RK, Peters C, Lorenz IC, Lindenbach BD (2009) Hepatitis C virus NS2 protein contributes to virus particle assembly via opposing epistatic interactions with the E1-E2 glycoprotein and NS3-NS4A enzyme complexes. *J Virol* 83: 8379-8395.
174. Counihan NA, Rawlinson SM, Lindenbach BD (2011) Trafficking of hepatitis C virus core protein during virus particle assembly. *PLoS Pathog* 7: e1002302.
175. Huang H, Sun F, Owen DM, Li W, Chen Y, et al. (2007) Hepatitis C virus production by human hepatocytes dependent on assembly and secretion of very low-density lipoproteins. *Proc Natl Acad Sci U S A* 104: 5848-5853.
176. Vieyres G, Thomas X, Descamps V, Duverlie G, Patel AH, et al. (2010) Characterization of the envelope glycoproteins associated with infectious hepatitis C virus. *J Virol* 84: 10159-10168.
177. Krey T, Thiel HJ, Rumenapf T (2005) Acid-resistant bovine pestivirus requires activation for pH-triggered fusion during entry. *J Virol* 79: 4191-4200.
178. Wozniak AL, Griffin S, Rowlands D, Harris M, Yi M, et al. (2010) Intracellular proton conductance of the hepatitis C virus p7 protein and its contribution to infectious virus production. *PLoS Pathog* 6: e1001087.
179. Welsch S, Muller B, Krausslich HG (2007) More than one door - Budding of enveloped viruses through cellular membranes. *FEBS Lett* 581: 2089-2097.
180. Ariumi Y, Kuroki M, Maki M, Ikeda M, Dansako H, et al. (2011) The ESCRT system is required for hepatitis C virus production. *PLoS One* 6: e14517.
181. Corless L, Crump CM, Griffin SD, Harris M (2010) Vps4 and the ESCRT-III complex are required for the release of infectious hepatitis C virus particles. *J Gen Virol* 91: 362-372.
182. Tamai K, Shiina M, Tanaka N, Nakano T, Yamamoto A, et al. (2012) Regulation of hepatitis C virus secretion by the Hrs-dependent exosomal pathway. *Virology* 422: 377-385.
183. Lai CK, Jeng KS, Machida K, Lai MM (2010) Hepatitis C virus egress and release depend on endosomal trafficking of core protein. *J Virol* 84: 11590-11598.
184. Timpe JM, Stamataki Z, Jennings A, Hu K, Farquhar MJ, et al. (2008) Hepatitis C virus cell-cell transmission in hepatoma cells in the presence of neutralizing antibodies. *Hepatology* 47: 17-24.
185. Grebely J, Prins M, Hellard M, Cox AL, Osburn WO, et al. (2012) Hepatitis C virus clearance, reinfection, and persistence, with insights from studies of injecting drug users: towards a vaccine. *Lancet Infect Dis* 12: 408-414.
186. Mosley JW, Operskalski EA, Tobler LH, Andrews WW, Phelps B, et al. (2005) Viral and host factors in early hepatitis C virus infection. *Hepatology* 42: 86-92.
187. Hoofnagle JH (1997) Hepatitis C: the clinical spectrum of disease. *Hepatology* 26: 15S-20S.
188. Cox AL, Netski DM, Mosbrugger T, Sherman SG, Strathdee S, et al. (2005) Prospective evaluation of community-acquired acute-phase hepatitis C virus infection. *Clin Infect Dis* 40: 951-958.

189. Wiegand J, Deterding K, Cornberg M, Wedemeyer H (2008) Treatment of acute hepatitis C: the success of monotherapy with (pegylated) interferon alpha. *J Antimicrob Chemother* 62: 860-865.
190. Maasoumy B, Wedemeyer H (2012) Natural history of acute and chronic hepatitis C. *Best Pract Res Clin Gastroenterol* 26: 401-412.
191. Bakr I, Rekacewicz C, El Hosseiny M, Ismail S, El Daly M, et al. (2006) Higher clearance of hepatitis C virus infection in females compared with males. *Gut* 55: 1183-1187.
192. Vogt M, Lang T, Frosner G, Klingler C, Sendl AF, et al. (1999) Prevalence and clinical outcome of hepatitis C infection in children who underwent cardiac surgery before the implementation of blood-donor screening. *N Engl J Med* 341: 866-870.
193. Rerksupphol S, Hardikar W, Dore GJ (2004) Long-term outcome of vertically acquired and post-transfusion hepatitis C infection in children. *J Gastroenterol Hepatol* 19: 1357-1362.
194. Bowen DG, Walker CM (2005) Adaptive immune responses in acute and chronic hepatitis C virus infection. *Nature* 436: 946-952.
195. Jinushi M, Takehara T, Tatsumi T, Kanto T, Miyagi T, et al. (2004) Negative regulation of NK cell activities by inhibitory receptor CD94/NKG2A leads to altered NK cell-induced modulation of dendritic cell functions in chronic hepatitis C virus infection. *J Immunol* 173: 6072-6081.
196. Ahlenstiel G, Martin MP, Gao X, Carrington M, Rehermann B (2008) Distinct KIR/HLA compound genotypes affect the kinetics of human antiviral natural killer cell responses. *J Clin Invest* 118: 1017-1026.
197. Wang CC, Krantz E, Klarquist J, Krows M, McBride L, et al. (2007) Acute hepatitis C in a contemporary US cohort: modes of acquisition and factors influencing viral clearance. *J Infect Dis* 196: 1474-1482.
198. Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, et al. (2009) Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 461: 798-801.
199. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, et al. (2009) Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 461: 399-401.
200. Seeff LB, Hoofnagle JH (2002) National Institutes of Health Consensus Development Conference: management of hepatitis C: 2002. *Hepatology* 36: S1-2.
201. Nelson DR (2001) The immunopathogenesis of hepatitis C virus infection. *Clin Liver Dis* 5: 931-953.
202. Bjoro K, Froland SS, Yun Z, Samdal HH, Haaland T (1994) Hepatitis C infection in patients with primary hypogammaglobulinemia after treatment with contaminated immune globulin. *N Engl J Med* 331: 1607-1611.
203. Walters KA, Syder AJ, Lederer SL, Diamond DL, Paepers B, et al. (2009) Genomic analysis reveals a potential role for cell cycle perturbation in HCV-mediated apoptosis of cultured hepatocytes. *PLoS Pathog* 5: e1000269.
204. Joyce MA, Walters KA, Lamb SE, Yeh MM, Zhu LF, et al. (2009) HCV induces oxidative and ER stress, and sensitizes infected cells to apoptosis in SCID/Alb-uPA mice. *PLoS Pathog* 5: e1000291.
205. Fillon S, Klingel K, Warntges S, Sauter M, Gabrysch S, et al. (2002) Expression of the serine/threonine kinase hSGK1 in chronic viral hepatitis. *Cell Physiol Biochem* 12: 47-54.
206. Kitamura Y, Ninomiya H (2003) Smad expression of hepatic stellate cells in liver cirrhosis in vivo and hepatic stellate cell line in vitro. *Pathol Int* 53: 18-26.
207. Joyce MA, Tyrrell DL (2010) The cell biology of hepatitis C virus. *Microbes Infect* 12: 263-271.

208. Thein HH, Yi Q, Dore GJ, Krahn MD (2008) Estimation of stage-specific fibrosis progression rates in chronic hepatitis C virus infection: a meta-analysis and meta-regression. *Hepatology* 48: 418-431.
209. Liang TJ, Heller T (2004) Pathogenesis of hepatitis C-associated hepatocellular carcinoma. *Gastroenterology* 127: S62-71.
210. Moreira RK (2007) Hepatic stellate cells and liver fibrosis. *Arch Pathol Lab Med* 131: 1728-1734.
211. Lok AS, Seeff LB, Morgan TR, di Bisceglie AM, Sterling RK, et al. (2009) Incidence of hepatocellular carcinoma and associated risk factors in hepatitis C-related advanced liver disease. *Gastroenterology* 136: 138-148.
212. Block TM, Mehta AS, Fimmel CJ, Jordan R (2003) Molecular viral oncology of hepatocellular carcinoma. *Oncogene* 22: 5093-5107.
213. Ray RB, Lagging LM, Meyer K, Ray R (1996) Hepatitis C virus core protein cooperates with ras and transforms primary rat embryo fibroblasts to tumorigenic phenotype. *J Virol* 70: 4438-4443.
214. Sakamuro D, Furukawa T, Takegami T (1995) Hepatitis C virus nonstructural protein NS3 transforms NIH 3T3 cells. *J Virol* 69: 3893-3896.
215. Park JS, Yang JM, Min MK (2000) Hepatitis C virus nonstructural protein NS4B transforms NIH3T3 cells in cooperation with the Ha-ras oncogene. *Biochem Biophys Res Commun* 267: 581-587.
216. Ghosh AK, Steele R, Meyer K, Ray R, Ray RB (1999) Hepatitis C virus NS5A protein modulates cell cycle regulatory genes and promotes cell growth. *J Gen Virol* 80 ( Pt 5): 1179-1183.
217. Potthoff A, Manns MP, Wedemeyer H (2010) Treatment of HBV/HCV coinfection. *Expert Opin Pharmacother* 11: 919-928.
218. Sugawara Y, Makuuchi M, Kato N, Shimotohno K, Takada K (1999) Enhancement of hepatitis C virus replication by Epstein-Barr virus-encoded nuclear antigen 1. *EMBO J* 18: 5755-5760.
219. Sugawara Y, Makuuchi M, Takada K (2000) Detection of Epstein-Barr virus DNA in hepatocellular carcinoma tissues from hepatitis C-positive patients. *Scand J Gastroenterol* 35: 981-984.
220. Kumar V, Kato N, Urabe Y, Takahashi A, Muroyama R, et al. (2011) Genome-wide association study identifies a susceptibility locus for HCV-induced hepatocellular carcinoma. *Nat Genet* 43: 455-458.
221. Zignego AL, Ferri C, Pileri SA, Caini P, Bianchi FB, et al. (2007) Extrahepatic manifestations of Hepatitis C Virus infection: a general overview and guidelines for a clinical approach. *Dig Liver Dis* 39: 2-17.
222. Cacoub P, Poynard T, Ghillani P, Charlotte F, Olivi M, et al. (1999) Extrahepatic manifestations of chronic hepatitis C. MULTIVIRC Group. Multidepartment Virus C. *Arthritis Rheum* 42: 2204-2212.
223. Sene D, Limal N, Cacoub P (2004) Hepatitis C virus-associated extrahepatic manifestations: a review. *Metab Brain Dis* 19: 357-381.
224. White DL, Ratzin V, El-Serag HB (2008) Hepatitis C infection and risk of diabetes: a systematic review and meta-analysis. *J Hepatol* 49: 831-844.
225. Mizorogi F, Hiramoto J, Nozato A, Takekuma Y, Nagayama K, et al. (2000) Hepatitis C virus infection in patients with B-cell non-Hodgkin's lymphoma. *Intern Med* 39: 112-117.
226. Pham TN, Michalak TI (2008) Occult persistence and lymphotropism of hepatitis C virus infection. *World J Gastroenterol* 14: 2789-2793.



227. Roque-Afonso AM, Ducoulombier D, Di Liberto G, Kara R, Gigou M, et al. (2005) Compartmentalization of hepatitis C virus genotypes between plasma and peripheral blood mononuclear cells. *J Virol* 79: 6349-6357.
228. Sarhan MA, Pham TN, Chen AY, Michalak TI (2012) Hepatitis C virus infection of human T lymphocytes is mediated by CD5. *J Virol* 86: 3723-3735.
229. Lee WM, Polson JE, Carney DS, Sahin B, Gale M, Jr. (2005) Reemergence of hepatitis C virus after 8.5 years in a patient with hypogammaglobulinemia: evidence for an occult viral reservoir. *J Infect Dis* 192: 1088-1092.
230. Mehta SH, Cox A, Hoover DR, Wang XH, Mao Q, et al. (2002) Protection against persistence of hepatitis C. *Lancet* 359: 1478-1483.
231. Lin A, Thadareddy A, Goldstein MJ, Lake-Bakaar G (2008) Immune suppression leading to hepatitis C virus re-emergence after sustained virological response. *J Med Virol* 80: 1720-1722.
232. Thomopoulos K, Giannakoulas NC, Tsamandas AC, Mimidis K, Fragopanagou E, et al. (2008) Recurrence of HCV infection in a sustained responder after chemotherapy for non-Hodgkin's lymphoma: successful retreatment. *Am J Med Sci* 336: 73-76.
233. Sarhan MA, Chen AY, Russell RS, Michalak TI (2012) Patient-derived hepatitis C virus and JFH-1 clones differ in their ability to infect human hepatoma cells and lymphocytes. *J Gen Virol* 93: 2399-2407.
234. Ducoulombier D, Roque-Afonso AM, Di Liberto G, Penin F, Kara R, et al. (2004) Frequent compartmentalization of hepatitis C virus variants in circulating B cells and monocytes. *Hepatology* 39: 817-825.
235. Pham TN, King D, Macparland SA, McGrath JS, Reddy SB, et al. (2008) Hepatitis C virus replicates in the same immune cell subsets in chronic hepatitis C and occult infection. *Gastroenterology* 134: 812-822.
236. Navas S, Martin J, Quiroga JA, Castillo I, Carreno V (1998) Genetic diversity and tissue compartmentalization of the hepatitis C virus genome in blood mononuclear cells, liver, and serum from chronic hepatitis C patients. *J Virol* 72: 1640-1646.
237. Laporte J, Bain C, Maurel P, Inchauspe G, Agut H, et al. (2003) Differential distribution and internal translation efficiency of hepatitis C virus quasispecies present in dendritic and liver cells. *Blood* 101: 52-57.
238. Pham TN, Coffin CS, Michalak TI (2010) Occult hepatitis C virus infection: what does it mean? *Liver Int* 30: 502-511.
239. Horner SM, Gale M, Jr. (2013) Regulation of hepatic innate immunity by hepatitis C virus. *Nat Med* 19: 879-888.
240. Li XD, Sun L, Seth RB, Pineda G, Chen ZJ (2005) Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. *Proc Natl Acad Sci U S A* 102: 17717-17722.
241. Loo YM, Gale M, Jr. (2011) Immune signaling by RIG-I-like receptors. *Immunity* 34: 680-692.
242. Takeuchi O, Akira S (2009) Innate immunity to virus infection. *Immunol Rev* 227: 75-86.
243. Noguchi T, Satoh S, Noshi T, Hatada E, Fukuda R, et al. (2001) Effects of mutation in hepatitis C virus nonstructural protein 5A on interferon resistance mediated by inhibition of PKR kinase activity in mammalian cells. *Microbiol Immunol* 45: 829-840.
244. Gale MJ, Jr., Korth MJ, Tang NM, Tan SL, Hopkins DA, et al. (1997) Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. *Virology* 230: 217-227.
245. Taylor DR, Shi ST, Romano PR, Barber GN, Lai MM (1999) Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. *Science* 285: 107-110.

246. Hosui A, Ohkawa K, Ishida H, Sato A, Nakanishi F, et al. (2003) Hepatitis C virus core protein differently regulates the JAK-STAT signaling pathway under interleukin-6 and interferon-gamma stimuli. *J Biol Chem* 278: 28562-28571.
247. Basu A, Meyer K, Ray RB, Ray R (2001) Hepatitis C virus core protein modulates the interferon-induced transacting factors of Jak/Stat signaling pathway but does not affect the activation of downstream IRF-1 or 561 gene. *Virology* 288: 379-390.
248. von Hahn T, Yoon JC, Alter H, Rice CM, Rehermann B, et al. (2007) Hepatitis C virus continuously escapes from neutralizing antibody and T-cell responses during chronic infection in vivo. *Gastroenterology* 132: 667-678.
249. Farci P, Shimoda A, Coiana A, Diaz G, Peddis G, et al. (2000) The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. *Science* 288: 339-344.
250. Timm J, Lauer GM, Kavanagh DG, Sheridan I, Kim AY, et al. (2004) CD8 epitope escape and reversion in acute HCV infection. *J Exp Med* 200: 1593-1604.
251. Seifert U, Liermann H, Racanelli V, Halenius A, Wiese M, et al. (2004) Hepatitis C virus mutation affects proteasomal epitope processing. *J Clin Invest* 114: 250-259.
252. Chang KM, Rehermann B, McHutchison JG, Pasquinelli C, Southwood S, et al. (1997) Immunological significance of cytotoxic T lymphocyte epitope variants in patients chronically infected by the hepatitis C virus. *J Clin Invest* 100: 2376-2385.
253. Urbani S, Amadei B, Tola D, Massari M, Schivazappa S, et al. (2006) PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion. *J Virol* 80: 11398-11403.
254. Nakamoto N, Kaplan DE, Coleclough J, Li Y, Valiga ME, et al. (2008) Functional restoration of HCV-specific CD8 T cells by PD-1 blockade is defined by PD-1 expression and compartmentalization. *Gastroenterology* 134: 1927-1937, 1937 e1921-1922.
255. Nakamoto N, Cho H, Shaked A, Olthoff K, Valiga ME, et al. (2009) Synergistic reversal of intrahepatic HCV-specific CD8 T cell exhaustion by combined PD-1/CTLA-4 blockade. *PLoS Pathog* 5: e1000313.
256. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R (2003) Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* 77: 4911-4927.
257. Wedemeyer H, He XS, Nascimbeni M, Davis AR, Greenberg HB, et al. (2002) Impaired effector function of hepatitis C virus-specific CD8<sup>+</sup> T cells in chronic hepatitis C virus infection. *J Immunol* 169: 3447-3458.
258. Urbani S, Amadei B, Fisicaro P, Tola D, Orlandini A, et al. (2006) Outcome of acute hepatitis C is related to virus-specific CD4 function and maturation of antiviral memory CD8 responses. *Hepatology* 44: 126-139.
259. Golden-Mason L, Castelblanco N, O'Farrelly C, Rosen HR (2007) Phenotypic and functional changes of cytotoxic CD56pos natural T cells determine outcome of acute hepatitis C virus infection. *J Virol* 81: 9292-9298.
260. Fernandez J, Taylor D, Morhardt DR, Mihalik K, Puig M, et al. (2004) Long-term persistence of infection in chimpanzees inoculated with an infectious hepatitis C virus clone is associated with a decrease in the viral amino acid substitution rate and low levels of heterogeneity. *J Virol* 78: 9782-9789.
261. Wolfl M, Rutebemberwa A, Mosbrugger T, Mao Q, Li HM, et al. (2008) Hepatitis C virus immune escape via exploitation of a hole in the T cell repertoire. *J Immunol* 181: 6435-6446.
262. McHutchison JG, Gordon SC, Schiff ER, Shiffman ML, Lee WM, et al. (1998) Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N Engl J Med* 339: 1485-1492.

263. Feld JJ, Hoofnagle JH (2005) Mechanism of action of interferon and ribavirin in treatment of hepatitis C. *Nature* 436: 967-972.
264. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, et al. (2001) Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 358: 958-965.
265. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, et al. (2002) Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 347: 975-982.
266. Hauser P (2004) Neuropsychiatric side effects of HCV therapy and their treatment: focus on IFN alpha-induced depression. *Gastroenterol Clin North Am* 33: S35-50.
267. Vermehren J, Sarrazin C (2012) The role of resistance in HCV treatment. *Best Pract Res Clin Gastroenterol* 26: 487-503.
268. Clark VC, Peter JA, Nelson DR (2013) New therapeutic strategies in HCV: second-generation protease inhibitors. *Liver Int* 33 Suppl 1: 80-84.
269. Lawitz E, Gane EJ (2013) Sofosbuvir for previously untreated chronic hepatitis C infection. *N Engl J Med* 369: 678-679.
270. Jacobson IM, Gordon SC, Kowdley KV, Yoshida EM, Rodriguez-Torres M, et al. (2013) Sofosbuvir for hepatitis C genotype 2 or 3 in patients without treatment options. *N Engl J Med* 368: 1867-1877.
271. Gao M, Nettles RE, Belema M, Snyder LB, Nguyen VN, et al. (2010) Chemical genetics strategy identifies an HCV NS5A inhibitor with a potent clinical effect. *Nature* 465: 96-100.
272. Scheel TK, Gottwein JM, Mikkelsen LS, Jensen TB, Bukh J (2011) Recombinant HCV variants with NS5A from genotypes 1-7 have different sensitivities to an NS5A inhibitor but not interferon-alpha. *Gastroenterology* 140: 1032-1042.
273. Guedj J, Dahari H, Rong L, Sansone ND, Nettles RE, et al. (2013) Modeling shows that the NS5A inhibitor daclatasvir has two modes of action and yields a shorter estimate of the hepatitis C virus half-life. *Proc Natl Acad Sci U S A* 110: 3991-3996.
274. Lok AS, Gardiner DF, Lawitz E, Martorell C, Everson GT, et al. (2012) Preliminary study of two antiviral agents for hepatitis C genotype 1. *N Engl J Med* 366: 216-224.
275. Lemon SM, McKeating JA, Pietschmann T, Frick DN, Glenn JS, et al. (2010) Development of novel therapies for hepatitis C. *Antiviral Res* 86: 79-92.
276. Syder AJ, Lee H, Zeisel MB, Grove J, Soulier E, et al. (2011) Small molecule scavenger receptor B1 antagonists are potent HCV entry inhibitors. *J Hepatol* 54: 48-55.
277. Zhu H, Wong-Staal F, Lee H, Syder A, McKelvy J, et al. (2012) Evaluation of ITX 5061, a scavenger receptor B1 antagonist: resistance selection and activity in combination with other hepatitis C virus antivirals. *J Infect Dis* 205: 656-662.
278. Paeshuyse J, Kaul A, De Clercq E, Rosenwirth B, Dumont JM, et al. (2006) The non-immunosuppressive cyclosporin DEBIO-025 is a potent inhibitor of hepatitis C virus replication in vitro. *Hepatology* 43: 761-770.
279. Flisiak R, Feinman SV, Jablkowski M, Horban A, Kryczka W, et al. (2009) The cyclophilin inhibitor Debio 025 combined with PEG IFNalpha2a significantly reduces viral load in treatment-naïve hepatitis C patients. *Hepatology* 49: 1460-1468.
280. Scheel TK, Rice CM (2013) Understanding the hepatitis C virus life cycle paves the way for highly effective therapies. *Nat Med* 19: 837-849.
281. Machlin ES, Sarnow P, Sagan SM (2011) Masking the 5' terminal nucleotides of the hepatitis C virus genome by an unconventional microRNA-target RNA complex. *Proc Natl Acad Sci U S A* 108: 3193-3198.
282. Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. *Cell* 136: 215-233.

283. Li Y, Masaki T, Yamane D, McGivern DR, Lemon SM (2013) Competing and noncompeting activities of miR-122 and the 5' exonuclease Xrn1 in regulation of hepatitis C virus replication. *Proc Natl Acad Sci U S A* 110: 1881-1886.
284. Shimakami T, Yamane D, Jangra RK, Kempf BJ, Spaniel C, et al. (2012) Stabilization of hepatitis C virus RNA by an Ago2-miR-122 complex. *Proc Natl Acad Sci U S A* 109: 941-946.
285. Lanford RE, Hildebrandt-Eriksen ES, Petri A, Persson R, Lindow M, et al. (2010) Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science* 327: 198-201.
286. Janssen HL, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, et al. (2013) Treatment of HCV infection by targeting microRNA. *N Engl J Med* 368: 1685-1694.
287. Farci P, Shimoda A, Wong D, Cabezon T, De Gioannis D, et al. (1996) Prevention of hepatitis C virus infection in chimpanzees by hyperimmune serum against the hypervariable region 1 of the envelope 2 protein. *Proc Natl Acad Sci U S A* 93: 15394-15399.
288. Schofield DJ, Bartosch B, Shimizu YK, Allander T, Alter HJ, et al. (2005) Human monoclonal antibodies that react with the E2 glycoprotein of hepatitis C virus and possess neutralizing activity. *Hepatology* 42: 1055-1062.
289. Folgori A, Capone S, Ruggeri L, Meola A, Sporeno E, et al. (2006) A T-cell HCV vaccine eliciting effective immunity against heterologous virus challenge in chimpanzees. *Nat Med* 12: 190-197.
290. Rollier C, Depla E, Drexhage JA, Verschoor EJ, Verstrepen BE, et al. (2004) Control of heterologous hepatitis C virus infection in chimpanzees is associated with the quality of vaccine-induced peripheral T-helper immune response. *J Virol* 78: 187-196.
291. Nascimbeni M, Mizukoshi E, Bosmann M, Major ME, Mihalik K, et al. (2003) Kinetics of CD4+ and CD8+ memory T-cell responses during hepatitis C virus rechallenge of previously recovered chimpanzees. *J Virol* 77: 4781-4793.
292. Bassett SE, Guerra B, Brasky K, Miskovsky E, Houghton M, et al. (2001) Protective immune response to hepatitis C virus in chimpanzees rechallenged following clearance of primary infection. *Hepatology* 33: 1479-1487.
293. Elbers K, Tautz N, Becher P, Stoll D, Rumenapf T, et al. (1996) Processing in the pestivirus E2-NS2 region: identification of proteins p7 and E2p7. *J Virol* 70: 4131-4135.
294. Murray CL, Jones CT, Rice CM (2008) Architects of assembly: roles of Flaviviridae non-structural proteins in virion morphogenesis. *Nat Rev Microbiol* 6: 699-708.
295. McLauchlan J, Lemberg MK, Hope G, Martoglio B (2002) Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. *EMBO J* 21: 3980-3988.
296. Carrere-Kremer S, Montpellier C, Lorenzo L, Brulin B, Cocquerel L, et al. (2004) Regulation of hepatitis C virus polyprotein processing by signal peptidase involves structural determinants at the p7 sequence junctions. *J Biol Chem* 279: 41384-41392.
297. Shanmugam S, Yi M (2013) The Efficiency of E2-p7 Processing Modulates the Production of Infectious Hepatitis C Virus. *J Virol*.
298. Stocks CE, Lobigs M (1998) Signal peptidase cleavage at the flavivirus C-prM junction: dependence on the viral NS2B-3 protease for efficient processing requires determinants in C, the signal peptide, and prM. *J Virol* 72: 2141-2149.
299. Griffin S, Clarke D, McCormick C, Rowlands D, Harris M (2005) Signal peptide cleavage and internal targeting signals direct the hepatitis C virus p7 protein to distinct intracellular membranes. *J Virol* 79: 15525-15536.

300. Jones CT, Murray CL, Eastman DK, Tassello J, Rice CM (2007) Hepatitis C virus p7 and NS2 proteins are essential for production of infectious virus. *J Virol* 81: 8374-8383.
301. Brohm C, Steinmann E, Friesland M, Lorenz IC, Patel A, et al. (2009) Characterization of determinants important for hepatitis C virus p7 function in morphogenesis by using trans-complementation. *J Virol* 83: 11682-11693.
302. Carrere-Kremer S, Montpellier-Pala C, Cocquerel L, Wychowski C, Penin F, et al. (2002) Subcellular localization and topology of the p7 polypeptide of hepatitis C virus. *J Virol* 76: 3720-3730.
303. Griffin SD, Harvey R, Clarke DS, Barclay WS, Harris M, et al. (2004) A conserved basic loop in hepatitis C virus p7 protein is required for amantadine-sensitive ion channel activity in mammalian cells but is dispensable for localization to mitochondria. *J Gen Virol* 85: 451-461.
304. Demaurex N, Distelhorst C (2003) Cell biology. Apoptosis--the calcium connection. *Science* 300: 65-67.
305. Haqshenas G, Mackenzie JM, Dong X, Gowans EJ (2007) Hepatitis C virus p7 protein is localized in the endoplasmic reticulum when it is encoded by a replication-competent genome. *J Gen Virol* 88: 134-142.
306. Vieyres G, Brohm C, Friesland M, Gentzsch J, Wolk B, et al. (2013) Subcellular Localization and Function of an Epitope-Tagged p7 Viroporin in Hepatitis C Virus-Producing Cells. *J Virol* 87: 1664-1678.
307. Harada T, Tautz N, Thiel HJ (2000) E2-p7 region of the bovine viral diarrhea virus polypeptide: processing and functional studies. *J Virol* 74: 9498-9506.
308. Griffin SD, Beales LP, Clarke DS, Worsfold O, Evans SD, et al. (2003) The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, Amantadine. *FEBS Lett* 535: 34-38.
309. Gonzalez ME, Carrasco L (2003) Viroporins. *FEBS Lett* 552: 28-34.
310. Nieva JL, Madan V, Carrasco L (2012) Viroporins: structure and biological functions. *Nat Rev Microbiol* 10: 563-574.
311. Wang K, Xie S, Sun B (2011) Viral proteins function as ion channels. *Biochim Biophys Acta* 1808: 510-515.
312. Liang X, Li ZY (2010) Ion channels as antiviral targets. *Virol Sin* 25: 267-280.
313. Ruch TR, Machamer CE (2012) The coronavirus E protein: assembly and beyond. *Viruses* 4: 363-382.
314. Kelly ML, Cook JA, Brown-Augsburger P, Heinz BA, Smith MC, et al. (2003) Demonstrating the intrinsic ion channel activity of virally encoded proteins. *FEBS Lett* 552: 61-67.
315. Schnell JR, Chou JJ (2008) Structure and mechanism of the M2 proton channel of influenza A virus. *Nature* 451: 591-595.
316. Pavlovic D, Neville DC, Argaud O, Blumberg B, Dwek RA, et al. (2003) The hepatitis C virus p7 protein forms an ion channel that is inhibited by long-alkyl-chain iminosugar derivatives. *Proc Natl Acad Sci U S A* 100: 6104-6108.
317. Durantel D, Branza-Nichita N, Carrouee-Durantel S, Butters TD, Dwek RA, et al. (2001) Study of the mechanism of antiviral action of iminosugar derivatives against bovine viral diarrhea virus. *J Virol* 75: 8987-8998.
318. Premkumar A, Wilson L, Ewart GD, Gage PW (2004) Cation-selective ion channels formed by p7 of hepatitis C virus are blocked by hexamethylene amiloride. *FEBS Lett* 557: 99-103.

319. StGelais C, Tuthill TJ, Clarke DS, Rowlands DJ, Harris M, et al. (2007) Inhibition of hepatitis C virus p7 membrane channels in a liposome-based assay system. *Antiviral Res* 76: 48-58.
320. StGelais C, Foster TL, Verow M, Atkins E, Fishwick CW, et al. (2009) Determinants of hepatitis C virus p7 ion channel function and drug sensitivity identified in vitro. *J Virol* 83: 7970-7981.
321. Meshkat Z, Audsley M, Beyer C, Gowans EJ, Haqshenas G (2009) Reverse genetic analysis of a putative, influenza virus M2 HXXXW-like motif in the p7 protein of hepatitis C virus. *J Viral Hepat* 16: 187-194.
322. Chew CF, Vijayan R, Chang J, Zitzmann N, Biggin PC (2009) Determination of pore-lining residues in the hepatitis C virus p7 protein. *Biophys J* 96: L10-12.
323. Foster TL, Verow M, Wozniak AL, Benthall MJ, Thompson J, et al. (2011) Resistance mutations define specific antiviral effects for inhibitors of the hepatitis C virus p7 ion channel. *Hepatology* 54: 79-90.
324. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, et al. (2005) Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 11: 791-796.
325. Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, et al. (2005) Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci U S A* 102: 9294-9299.
326. Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, et al. (2005) Complete replication of hepatitis C virus in cell culture. *Science* 309: 623-626.
327. Steinmann E, Whitfield T, Kallis S, Dwek RA, Zitzmann N, et al. (2007) Antiviral effects of amantadine and iminosugar derivatives against hepatitis C virus. *Hepatology* 46: 330-338.
328. Bergeron JJ, Brenner MB, Thomas DY, Williams DB (1994) Calnexin: a membrane-bound chaperone of the endoplasmic reticulum. *Trends Biochem Sci* 19: 124-128.
329. Branza-Nichita N, Durantel D, Carrouee-Durantel S, Dwek RA, Zitzmann N (2001) Antiviral effect of N-butyldeoxynojirimycin against bovine viral diarrhea virus correlates with misfolding of E2 envelope proteins and impairment of their association into E1-E2 heterodimers. *J Virol* 75: 3527-3536.
330. Fischer PB, Collin M, Karlsson GB, James W, Butters TD, et al. (1995) The alpha-glucosidase inhibitor N-butyldeoxynojirimycin inhibits human immunodeficiency virus entry at the level of post-CD4 binding. *J Virol* 69: 5791-5797.
331. Premkumar A, Dong X, Haqshenas G, Gage PW, Gowans EJ (2006) Amantadine inhibits the function of an ion channel encoded by GB virus B, but fails to inhibit virus replication. *Antivir Ther* 11: 289-295.
332. Griffin S, Stgelais C, Owsianka AM, Patel AH, Rowlands D, et al. (2008) Genotype-dependent sensitivity of hepatitis C virus to inhibitors of the p7 ion channel. *Hepatology* 48: 1779-1790.
333. Atkins E, Tatineni R, Li H, Gretch D, Harris M, et al. (2014) The stability of secreted, acid-labile H77/JFH-1 hepatitis C virus (HCV) particles is altered by patient isolate genotype 1a p7 sequences. *Virology* 448: 117-124.
334. Khoury G, Ewart G, Luscombe C, Miller M, Wilkinson J (2010) Antiviral efficacy of the novel compound BIT225 against HIV-1 release from human macrophages. *Antimicrob Agents Chemother* 54: 835-845.
335. Wang YT, Hsu HJ, Fischer WB (2013) Computational modeling of the p7 monomer from HCV and its interaction with small molecule drugs. *Springerplus* 2: 324.
336. Luscombe CA, Huang Z, Murray MG, Miller M, Wilkinson J, et al. (2010) A novel Hepatitis C virus p7 ion channel inhibitor, BIT225, inhibits bovine viral diarrhea virus in vitro and

- shows synergism with recombinant interferon-alpha-2b and nucleoside analogues. *Antiviral Res* 86: 144-153.
337. Mihm U, Grigorian N, Welsch C, Herrmann E, Kronenberger B, et al. (2006) Amino acid variations in hepatitis C virus p7 and sensitivity to antiviral combination therapy with amantadine in chronic hepatitis C. *Antivir Ther* 11: 507-519.
  338. Castelain S, Bonte D, Penin F, Francois C, Capron D, et al. (2007) Hepatitis C Virus p7 membrane protein quasispecies variability in chronically infected patients treated with interferon and ribavirin, with or without amantadine. *J Med Virol* 79: 144-154.
  339. Lake-Bakaar G, Ruffini L, Kuzmic P (2003) Effect of ribavirin and amantadine on early hepatitis C virus RNA rebound and clearance in serum during daily high-dose interferon. *Dig Dis Sci* 48: 126-139.
  340. Montserret R, Saint N, Vanbelle C, Salvay AG, Simorre JP, et al. (2010) NMR structure and ion channel activity of the p7 protein from hepatitis C virus. *J Biol Chem* 285: 31446-31461.
  341. Clarke D, Griffin S, Beales L, Gelais CS, Burgess S, et al. (2006) Evidence for the formation of a heptameric ion channel complex by the hepatitis C virus p7 protein in vitro. *J Biol Chem* 281: 37057-37068.
  342. Patargias G, Zitzmann N, Dwek R, Fischer WB (2006) Protein-protein interactions: modeling the hepatitis C virus ion channel p7. *J Med Chem* 49: 648-655.
  343. Cook GA, Dawson LA, Tian Y, Opella SJ (2013) Three-Dimensional Structure and Interaction Studies of Hepatitis C Virus p7 in 1,2-Dihexanoyl-sn-glycero-3-phosphocholine by Solution Nuclear Magnetic Resonance. *Biochemistry*.
  344. Foster TL, Thompson GS, Kalverda AP, Kankanala J, Bentham M, et al. (2013) Structure-guided design affirms inhibitors of hepatitis C virus p7 as a viable class of antivirals targeting virion release. *Hepatology*.
  345. Luik P, Chew C, Aittoniemi J, Chang J, Wentworth P, Jr., et al. (2009) The 3-dimensional structure of a hepatitis C virus p7 ion channel by electron microscopy. *Proc Natl Acad Sci U S A* 106: 12712-12716.
  346. Chandler DE, Penin F, Schulten K, Chipot C (2012) The p7 protein of hepatitis C virus forms structurally plastic, minimalist ion channels. *PLoS Comput Biol* 8: e1002702.
  347. OuYang B, Xie S, Berardi MJ, Zhao X, Dev J, et al. (2013) Unusual architecture of the p7 channel from hepatitis C virus. *Nature* 498: 521-525.
  348. Sakai A, Claire MS, Faulk K, Govindarajan S, Emerson SU, et al. (2003) The p7 polypeptide of hepatitis C virus is critical for infectivity and contains functionally important genotype-specific sequences. *Proc Natl Acad Sci U S A* 100: 11646-11651.
  349. Steinmann E, Penin F, Kallis S, Patel AH, Bartenschlager R, et al. (2007) Hepatitis C virus p7 protein is crucial for assembly and release of infectious virions. *PLoS Pathog* 3: e103.
  350. Bartosch B, Dubuisson J, Cosset FL (2003) Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. *J Exp Med* 197: 633-642.
  351. Hsu M, Zhang J, Flint M, Logvinoff C, Cheng-Mayer C, et al. (2003) Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. *Proc Natl Acad Sci U S A* 100: 7271-7276.
  352. Flint M, Logvinoff C, Rice CM, McKeating JA (2004) Characterization of infectious retroviral pseudotype particles bearing hepatitis C virus glycoproteins. *J Virol* 78: 6875-6882.
  353. Blight KJ, McKeating JA, Rice CM (2002) Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J Virol* 76: 13001-13014.
  354. Lohmann V, Korner F, Koch J, Herian U, Theilmann L, et al. (1999) Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 285: 110-113.

355. Boson B, Granio O, Bartenschlager R, Cosset FL (2011) A concerted action of hepatitis C virus p7 and nonstructural protein 2 regulates core localization at the endoplasmic reticulum and virus assembly. *PLoS Pathog* 7: e1002144.
356. Tedbury P, Welbourn S, Pause A, King B, Griffin S, et al. (2011) The subcellular localization of the hepatitis C virus non-structural protein NS2 is regulated by an ion channel-independent function of the p7 protein. *J Gen Virol* 92: 819-830.
357. Popescu CI, Callens N, Trinel D, Roingeard P, Moradpour D, et al. (2011) NS2 protein of hepatitis C virus interacts with structural and non-structural proteins towards virus assembly. *PLoS Pathog* 7: e1001278.
358. Andre P, Komurian-Pradel F, Deforges S, Perret M, Berland JL, et al. (2002) Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. *J Virol* 76: 6919-6928.
359. Jones DM, Atoom AM, Zhang X, Kottlilil S, Russell RS (2011) A genetic interaction between the core and NS3 proteins of hepatitis C virus is essential for production of infectious virus. *J Virol* 85: 12351-12361.
360. Bentham MJ, Foster TL, McCormick C, Griffin S (2013) Mutations in hepatitis C virus p7 reduce both the egress and infectivity of assembled particles via impaired proton channel function. *J Gen Virol* 94: 2236-2248.
361. Lee A, Liu S, Wang T (2013) Identification of novel human kinases that suppress hepatitis C virus infection. *J Viral Hepat*.
362. Aweya JJ, Mak TM, Lim SG, Tan YJ (2013) The p7 protein of the hepatitis C virus induces cell death differently from the influenza A virus viroporin M2. *Virus Res* 172: 24-34.
363. Yi M, Ma Y, Yates J, Lemon SM (2007) Compensatory mutations in E1, p7, NS2, and NS3 enhance yields of cell culture-infectious intergenotypic chimeric hepatitis C virus. *J Virol* 81: 629-638.
364. Murray CL, Jones CT, Tassello J, Rice CM (2007) Alanine scanning of the hepatitis C virus core protein reveals numerous residues essential for production of infectious virus. *J Virol* 81: 10220-10231.
365. Russell RS, Meunier JC, Takikawa S, Faulk K, Engle RE, et al. (2008) Advantages of a single-cycle production assay to study cell culture-adaptive mutations of hepatitis C virus. *Proc Natl Acad Sci U S A* 105: 4370-4375.
366. Liang C, Rong L, Russell RS, Wainberg MA (2000) Deletion mutagenesis downstream of the 5' long terminal repeat of human immunodeficiency virus type 1 is compensated for by point mutations in both the U5 region and gag gene. *J Virol* 74: 6251-6261.
367. Rong L, Russell RS, Hu J, Guan Y, Kleiman L, et al. (2001) Hydrophobic amino acids in the human immunodeficiency virus type 1 p2 and nucleocapsid proteins can contribute to the rescue of deleted viral RNA packaging signals. *J Virol* 75: 7230-7243.
368. Russell RS, Hu J, Laughrea M, Wainberg MA, Liang C (2002) Deficient dimerization of human immunodeficiency virus type 1 RNA caused by mutations of the u5 RNA sequences. *Virology* 303: 152-163.
369. Bungyoku Y, Shoji I, Makine T, Adachi T, Hayashida K, et al. (2009) Efficient production of infectious hepatitis C virus with adaptive mutations in cultured hepatoma cells. *J Gen Virol* 90: 1681-1691.
370. Liu S, Yang W, Shen L, Turner JR, Coyne CB, et al. (2009) Tight junction proteins claudin-1 and occludin control hepatitis C virus entry and are downregulated during infection to prevent superinfection. *J Virol* 83: 2011-2014.
371. Tellier R, Bukh J, Emerson SU, Miller RH, Purcell RH (1996) Long PCR and its application to hepatitis viruses: amplification of hepatitis A, hepatitis B, and hepatitis C virus genomes. *J Clin Microbiol* 34: 3085-3091.



- 372. Zhong J, Gastaminza P, Chung J, Stamatakis Z, Isogawa M, et al. (2006) Persistent hepatitis C virus infection in vitro: coevolution of virus and host. *J Virol* 80: 11082-11093.
- 373. Delgrange D, Pillez A, Castelain S, Cocquerel L, Rouille Y, et al. (2007) Robust production of infectious viral particles in Huh-7 cells by introducing mutations in hepatitis C virus structural proteins. *J Gen Virol* 88: 2495-2503.
- 374. Kaul A, Woerz I, Meuleman P, Leroux-Roels G, Bartenschlager R (2007) Cell culture adaptation of hepatitis C virus and in vivo viability of an adapted variant. *J Virol* 81: 13168-13179.
- 375. Alsaleh K, Delavalle PY, Pillez A, Duverlie G, Descamps V, et al. (2010) Identification of basic amino acids at the N-terminal end of the core protein that are crucial for hepatitis C virus infectivity. *J Virol* 84: 12515-12528.
- 376. Bentham MJ, Foster TL, McCormick C, Griffin S (2013) Mutations in Hepatitis C virus p7 reduce both the egress and infectivity of assembled particles via impaired proton channel function. *J Gen Virol*.